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Effect of γ-amino Butyric Acid on Limpet Populations: Towards the Future Management and Conservation of Endangered Patellid Species

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Abstract Many neurotransmitters, such as γ-amino butyric acid (GABA), can act as chemical cues influencing settlement and metamorphosis in benthic marine invertebrates. This effect has been described especially in mollusks, such as mussels, clams, or haliotids. This study describes the first record of the effect of GABA on patellogastropod populations. Special attention was paid to the effect of the compound on recruitment processes. The experiment was carried out using 10×10 cm artificial limestone plates that were drilled into intertidal rocks at different inclinations, and periodically treated with a 1 mM GABA solution. A total of five limpet species was considered (four patellid limpet species and the pulmonate *Siphonaria pectinata*). Each individual recorded on the plates as well as within a 20×20 cm quadrant was measured, identified to species level, and its straight-line distance to the application point was registered. Treated surfaces were the first to possess both adults and recruits. Individuals also were found in a higher number around GABA-treated plates than around controls. The results indicated that the compound may not only enhance recruitment, but also might accelerate it. Recruits were located at higher distances from GABA treated plates than from control surfaces. This supports the hypothesis that this is the life history stage most sensitive to the compound. The behavior shown by the individuals belonging to the two endangered patellid species present in the study area (*Patella ferruginea* and *Cymbula nigra*) also were analyzed. For the former, the results indicated that the use of GABA may have similar effects on recruitment similar to the presence of adult conspecifics. This is the first report of the effect of GABA on patellid limpet recruitment and population dynamics. Conservational implications of the results are discussed.

Key Words Chemical cues · Larvae attraction · Patellid limpet · Recruitment · Endangered species · Patellidae

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Introduction

The life history of many benthic marine invertebrate species includes a planktonic phase, after which individuals settle on the substrate to start a new and less mobile life stage. This process, referred to as "settlement" throughout, is mediated not only by physical processes, but also by chemical cues in a wide variety of species (e.g., Morse et al., 1979a; Burke, 1984; Pearce and Scheibling, 1990; Swanson et al., 2004; García-Lavandeira et al., 2005). Later, the surviving "settlers", after reaching a size of 2–3 mm, become "recruits" and are then visible and can be studied further (for definitions see the review by Rodríguez et al., 1993). After some time (which depends on the species considered), recruits acquire a certain size/age when sexual maturity is achieved. These sexually mature individuals, become "adults".

The factors that influence settlement and the selection of substrates by larvae of benthic species have been studied for decades, as they can determine local densities and distribution (Grosberg, 1982; Keough and Downes, 1982; Butler, 1986; Sutherland, 1990). Hydrodynamic factors are important controllers of larvae recruitment in both soft-sediment (Eckman, 1983; Butman, 1987) and hard-bottom benthic species (Pawlik et al., 1991). Since the 1970s,

larvae recruitment as a response to chemical compounds produced by conspecifics (Burke, 1984), prey (Hadfield and Scheuer, 1985) and biofilms (Wieczorek and Todd, 1998) has been described in a wide range of invertebrates. Chemical compounds are capable of attracting the larvae of these species to conditions more favorable than if they settled indiscriminately (Thorson, 1950; Burke, 1983; Pawlik, 1990, 1992) and naturally produce a gregarious settlement. Such stimuli can enhance the chances for larvae survival, as falling or selecting a non-appropriate substrate and the impossibility of moving towards more favorable conditions can lead to the death of an individual, and eventually to the collapse of the population.

A great variety of larvae-attracting compounds have been reported, ranging from low molecular weight water-soluble compounds (Hadfield and Pennington, 1990; Gibson and Chia, 1994; Swanson et al., 2006) to high molecular weight carbohydrates (Morse and Morse, 1991; Krug and Manzi, 1999), proteins (Jensen and Morse, 1990) and lipids (Takahashi et al., 2002). Among these compounds, *L*-3,4-dihydroxyphenylalanine (Coon et al., 1985), epinephrine (Pearce and Scheibling, 1990), or histamine (Swanson et al., 2004) have been identified.

Gamma-amino butyric acid (GABA) ($C_4H_9NO_2$) is a bioactive molecule widely known for its function as a neurotransmitter. The effect of GABA as a settlement inducer has been studied in many gastropod species (Hadfield, 1984; García-Lavandeira et al., 2005; Mesías-Gansbiller et al., 2008). This effect was described first in abalones by Morse et al. (1979a). Haliotid species are important commercially, and factors that promote their settlement have been well studied (Morse et al., 1979b; Bryan and Qian, 1998; Li et al., 2006; Searcy-Bernal and Anguiano-Beltrán, 2007). Until 2008, GABA was considered to be an artificial inducer of settlement. However, it recently has been shown that GABA is naturally produced by abalone species and other mollusks (Laimek et al., 2008).

The main objective of the present study was to determine the effect of GABA on limpet recruitment. Our study site was the coast of Ceuta, a city located in North Africa (Strait of Gibraltar) where a high diversity of limpet species is found: the pulmonate *Siphonaria pectinata* and patellid limpets such as *Patella caerulea, P. rustica*, or *P. ulyssiponensis*. In this area, we additionally find important populations of two endangered species: *P. ferruginea* and *Cymbula nigra*. Even though *P. ferruginea* generally was distributed throughout the Western Mediterranean basin during the Pleistocene, since the beginning of the 20th century, its population has suffered a clear reduction in number (Laborel-Deguen and Laborel, 1991; Templado, 2001) mainly because of human exploitation (Aversano, 1986; Guerra-García et al., 2004; Moreno, 2004). It is because of this situation that the species has been considered as one of the most endangered invertebrate species in Europe. Recently, the Spanish National Strategy for the Conservation of the species was developed (MMAMRM, 2008), which establishes several management measures and conservation proposals. One of these measures is the development of adequate protocols for the translocation of individuals for repopulation purposes. Among these protocols, we can find some proposals as the collection of recruits through experimental plates, which could be later translocated. It is in this context that the main objectives of the present study were established.

Methods and Materials

Objectives and Hypotheses The main objective of the present study was to determine how GABA affects limpet populations (especially recruitment). There is no available information regarding the influence of chemical cues on the settlement of patellid limpets. GABA was selected because it is a product known to influence such processes in a wide variety of gastropod species, and is naturally produced by similar species such as haliotids (Laimek et al., 2008). The study was conducted using two monitoring methodologies: a) documenting the colonization of plates in the intertidal zone that were or were not treated with GABA; b) analyzing the limpet community around the GABA-treated plates and comparing the results with those obtained around control (non-treated) areas. This allowed us to determine if the compound had some effect on limpet individuals surrounding treated areas and to infer generally on the scale of influence of GABA in the marine environment. Additionally, other parameters were taken into consideration: bi) the straight-line distance between the GABA application point on both adults and recruits to confirm that GABA was not repulsive and that adults were not also influencing settlement; bii) the effect of the presence of adult conspecifics on recruitment. Regarding this latter point, special attention was paid to the two protected limpet species located in the area: *P. ferruginea* and *C. nigra*. Both species naturally present a gregarious distribution (Espinosa et al., 2006; Rivera-Ingraham et al., in press). This may be the result of larvae preferentially settling on or near adult conspecifics, which could be explained by the fact that adults may produce naturally some kind of product that would result in larval attraction. Taking this into account, the effect of the presence of adults on recruitment in control areas was compared with the effect of GABA on recruitment where no adults are found.

Three different locations and three different substrate inclinations were used in the present study as fixed factors. Regarding the latter, all surfaces considered in the study

were classified as three different inclinations (horizontal= 0–20°, inclined=20–60°, and vertical=>60°), in order to assure that neither this factor nor location affected the results.

Experimental Design Patella ferruginea was chosen as a representative species for developing the experimental design since it is the most endangered patellid species in the area. It is an upper mid-littoral species (Espinosa, 2006) and shares the habitat with many other patellid limpets. Considering that the species is commonly present on artificial substrates (Guerra-García et al., 2004), three different artificial breakwaters in Ceuta were selected (Fig. 1): "Chorrillo", located in the city's South Bay and where great *P. ferruginea* densities can be found; "Foso de San Felipe", which constitutes the southern exit of Ceuta's commercial port (South Bay); and finally, "Parque del Mediterráneo", located inside the city's commercial port. The latter is completely closed to human access because it is private property and holds one of the most important *P. ferruginea* (Espinosa et al., 2009) and *C. nigra* (Rivera-Ingraham et al., in press) populations in terms of density and size distribution. These three locations were used as three different spatial replicates, in order to confirm that the results were not dependent on location.

In the present study, 10×10 cm limestone plates were used. The surface was modified so that it matched the heterogeneity of *P. ferruginea*'s natural habitat. In January 2009, 30 of these cement plates were fixed during low tide to the intertidal rocks of each area (a total of 90 plates), paying special attention that every plate was placed within *P. ferruginea*'s habitat. This was done by using a pneumatic drill connected to a scuba-diving tank to make the necessary holes. After inserting a rawl, the plate was fastened to the substrate by one screw located in the plate's center. A polycarbonate surface of the same size was placed between the plate and the rock in order to minimize the impact caused by waves. For each site, the 30 plates were arranged in five different replicas. Each replica was composed of two sets of 3 plates (6 plates per replica), each in a different inclination: vertical (>60°), inclined (60°–20°) and horizontal (<20°) (refer to Fig. 1 for more details).

A 1 mM γ-amino butyric acid, GABA (Sigma-Aldrich Chemie GmbH, Germany) stock solution was prepared in distilled water. This concentration was selected because recent findings have shown that other similar species (such as haliotids) present an average concentration of 0.68 mM GABA in the pedal mucus (Laimek et al., 2008). In any case, these values are significantly higher than those used in the laboratory and aquaculture conditions (Gapasin and Polohan, 2004), but in the present study, the solution was continuously dissolved and washed away by waves. After placing all plates, the solution was applied alternatively to one of the two subsets of each of the replicas. Our main hypothesis was that GABA may be present in the pedal mucus of limpets (as seen in *Haliotis* sp) (Laimek et al., 2008). Mucus has been found to persist around 5 d in *Patella vulgata* (Davies et al., 1992) and up to 2.5 wk in the case of *Littorina littorea* (Davies and Blackwell, 2007). Taking this into account, the application of the GABA solution was performed weekly during February and March 2009, coinciding with the *P. ferruginea* recruitment period (Espinosa, 2006). This task was carried out during low tide periods with the help of a paintbrush. In successive weeks, the solution was applied to the complete plate, even if animals were found on them. The solution was allowed to dry naturally on the plates, which only took some minutes thanks to the windy conditions predominant in the area. At the same time, all individuals located on the plates were identified and their maximum shell length was registered.

After 2 mo, no additional GABA was applied to the experimental surfaces. The registration of individuals located on the plates then was carried out monthly until September 2009. Additionally, pictures of every plate and the surrounding area were taken using a 20×20 quadrant (with the corresponding limestone plate located in its center) on three occasions: immediately after the last GABA application (T=0), 1 mo (T=1), and 2 mo (T=2) after the last GABA application (March, April, and May, 2009 respectively). This analysis was carried out in order to register the evolution of the composition of the population on the plates and adjacent areas after having treated the plates with GABA for 2 mo, and to show whether the population changed after application of the compound. These photographs then were used for further image analysis, in which each of the individuals located inside the 20×20 cm quadrant was registered and identified to the species level (when possible). Additionally, maximum shell length and minimum distance to the plate were measured for each individual. In order to achieve this, a matrix consisting of 50 quadrants was superimposed over each of the pictures taken using Photoshop 6.0 © (1989–2000 Adobe Systems Incorporated). This matrix served as a reference in order to make the counting of the individuals easier. With this same software, each individual was marked with the paintbrush tool. Additionally, images were saved in a bmp extension so they could be exported to Scion Image Alpha 4.0.3.2. © (2000–2001 Scion Corporation). With this software, and using a known distance as a reference (10 cm for our case, which was the total length of the limestone plate), the maximum shell length and minimum distance to the plate were recorded for each individual. Finally, all individuals

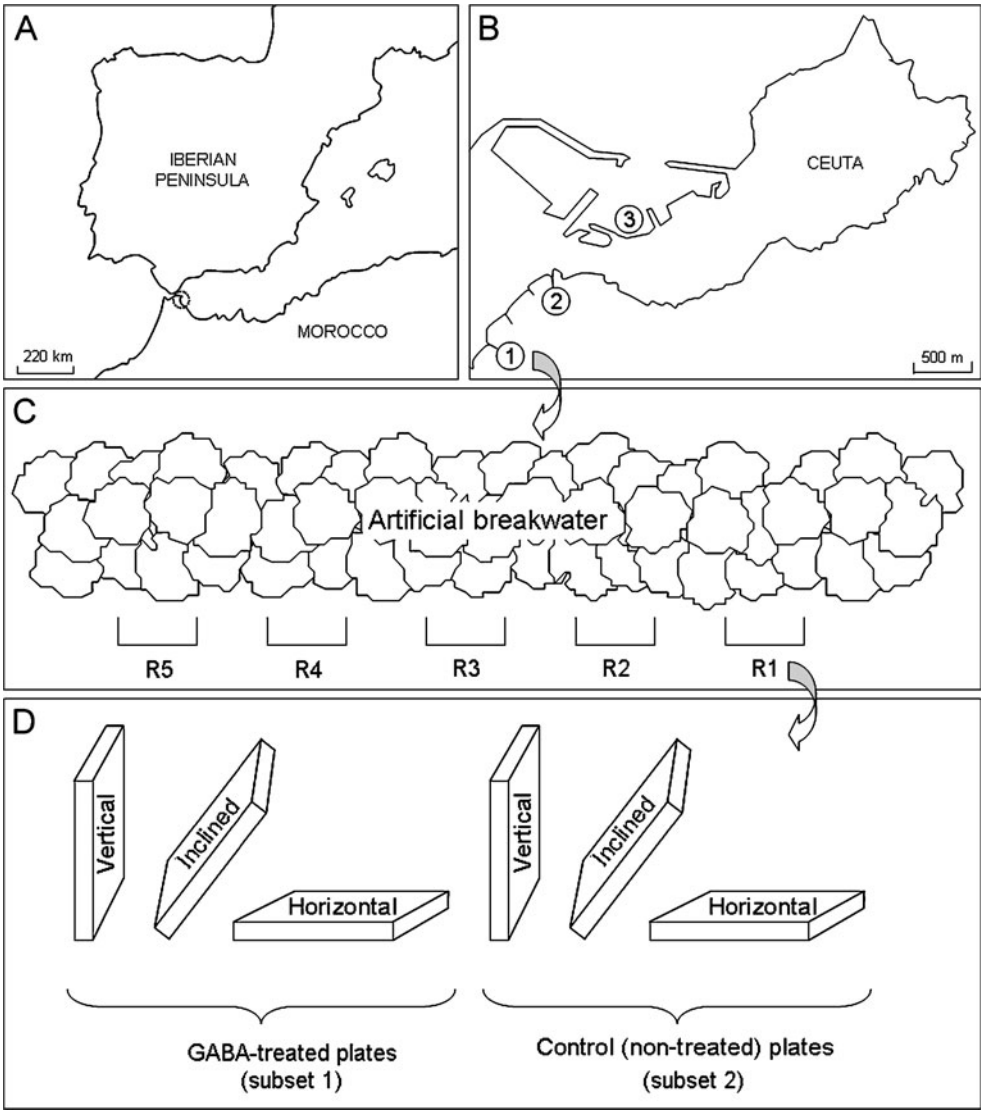


Fig. 1 Location of the study site. A.: Spain. B.: Ceuta, where 1: Chorrillo (South Bay); 2: Foso de San Felipe (South Bay); 3: Parque del Mediterráneo (North Bay). C.: General structure of the artificial breakwaters present in each location and distribution of the replicas (five replicas and 30 plates per location). D.: replica structure. Each replica was composed of 6 plates (divided in 2 subsets of 3 plates each). Each plate in a subset was fixed in a different inclination: horizontal (0–20°), inclined (20–60°) and vertical (>60°). One subset per replica was treated with the GABA solution

recorded on the plates and on the surrounding substrates were classified as adults or recruits, based on the size of first maturation recorded for the species: in this sense, *Siphonaria pectinata* individuals of less than 5 mm were considered recruits, based on the reports from Ocaña and Emson (1999); for the cases of the giant limpet species *P. ferruginea* and *C. nigra*, a limit of 20 mm was considered, while for *P. caerulea* and *P. rustica*, a limit of 10 mm was established (Frenkiel, 1975).

Statistical analyses were carried out with the SPSS 14.0 statistical package. One-way ANOVA tests were used in order to determine if there were significant differences between GABA-treated plates and non-treated plates, after confirming the compliance of the normal distribution (Kolmogorof-Smirnov test) and equal variance (Levene test) assumptions. For those cases where these conditions were not met, non-parametric analyses were carried out using the same statistical software.

Results

A) *Plate Colonization*. Results regarding this part of the study are presented in Fig. 2, which shows the frequency of individuals of each of the considered species per treatment and through time. It is interesting to note that the GABA plates were the first to register individuals, and *P. caerulea* adults were the first to colonize the plates. They were present three and five weeks into the experiment on GABA-treated plates and controls, respectively. Regarding recruits, *C. nigra* individuals were the first to be registered on both treated (time = +4 weeks) and control plates (time = +7 weeks). All *C. nigra* individuals recorded on the plates (a total of 12) were recruits, and four of them were obtained during the GABA application period. Returning to Fig. 2, we observe how, after the last GABA treatment, the number of *C. nigra* recruits significantly increased on treated

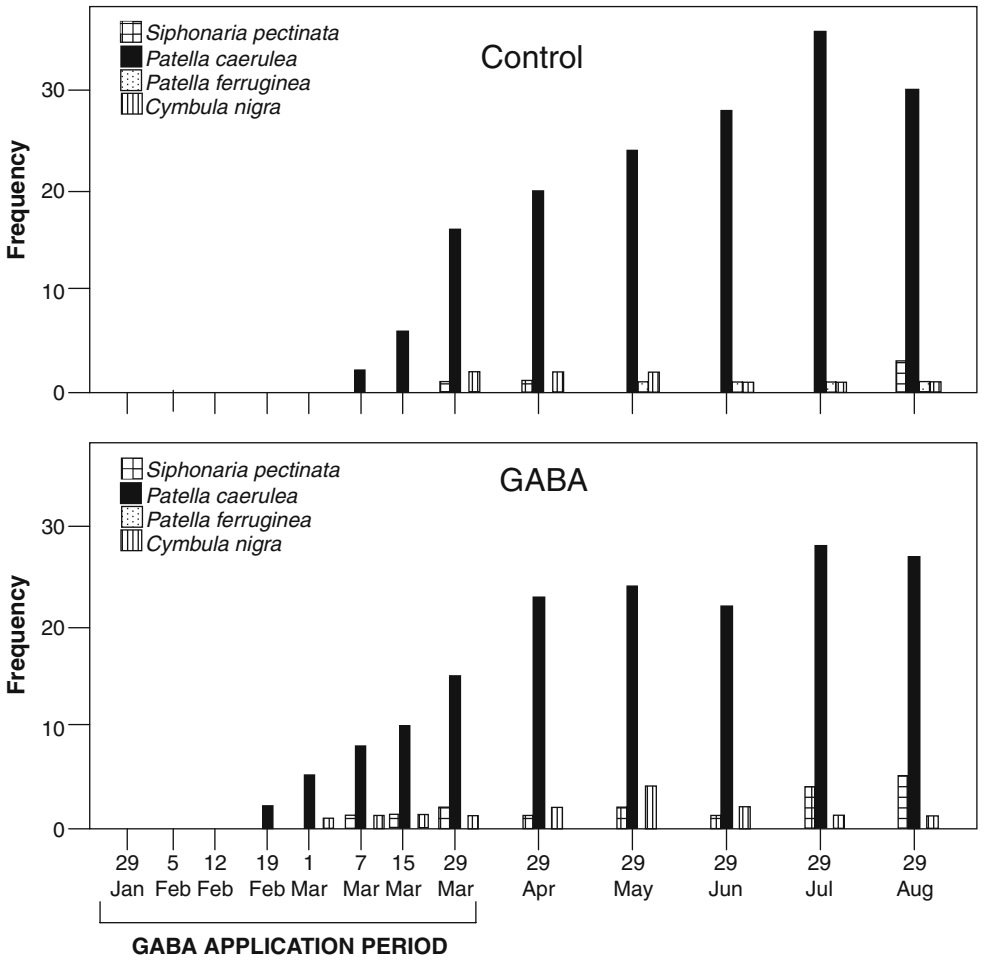


Fig. 2 Total number of individuals recorded over limestone plates, per species (*Siphonaria, Patella, Cymbula*), treatment, and sampling date. The experiment began at the end of January 2009 with the establishment of the plates in the intertidal zone and with the first GABA treatment. After that, the solution was applied to the plates weekly for two months

plates. A similar pattern was seen with *S. pectinata* recruits, which also experienced important growth in numbers during the last two months of the study, which was significantly greater on the treated plates. Regarding *P. ferruginea*, only one individual was located on the limestone plates. This individual was a recruit that settled on a non-treated plate in June, three months after the last application of GABA.

B) *Population Composition in Quadrants*. The total number of individuals found per quadrant around the treated plates was significantly higher (28.79± 15.16 ind) than around control areas (21.47± 14.78 ind) at T=0 (F=5.25; P=0.024). These differences were maintained through time, at T=1 (F=4.52; P=0.036) and T=2 (F=6.29; P=0.014).

As a general pattern, the average number of adults and recruits was usually higher around treated than control areas (adults: Chi^2=4.94; P<0.05; recruits: Chi^2= 7.08; P<0.01) (Fig. 3). However, there were some exceptions, such as the case of *P. rustica* recruits, which were present in higher numbers around the control areas over the entire duration of the experiment, although no significant differences were recorded between treatments. With respect to adults, it was interesting to note that only *P. caerulea* individuals showed differences between treatments, and were present in a significantly higher number around the GABA-treated plates. When considering the recruit fraction of the populations, the two endangered species (*P. ferruginea* and *C. nigra*) presented significantly greater numbers associated with GABA treatment. It also was noticeable how these differences disappeared at T=1 for *P. caerulea* and *C. nigra*, while they were maintained up to T=2 for *P. ferruginea* recruits. It was also noted that for the latter, while the number of recruits decreased at T=2, the number of adults did not rise, meaning that the loss of recruits was not due to the normal growth process of the individuals. Also noticeable was the case of *S. pectinata*, which presented no significant differences between treatments at T=0 and T=1, but presented a significantly greater number of recruits around the GABA-treated plates at T=2 (two months after the last application).

The distance of adults to the plates was not significant between treatments at T=0 (F=3.319; P=0.069). However, recruits presented significantly greater distances to treated plates than to controls, from T=0 (F=10.737; P=0.001) up

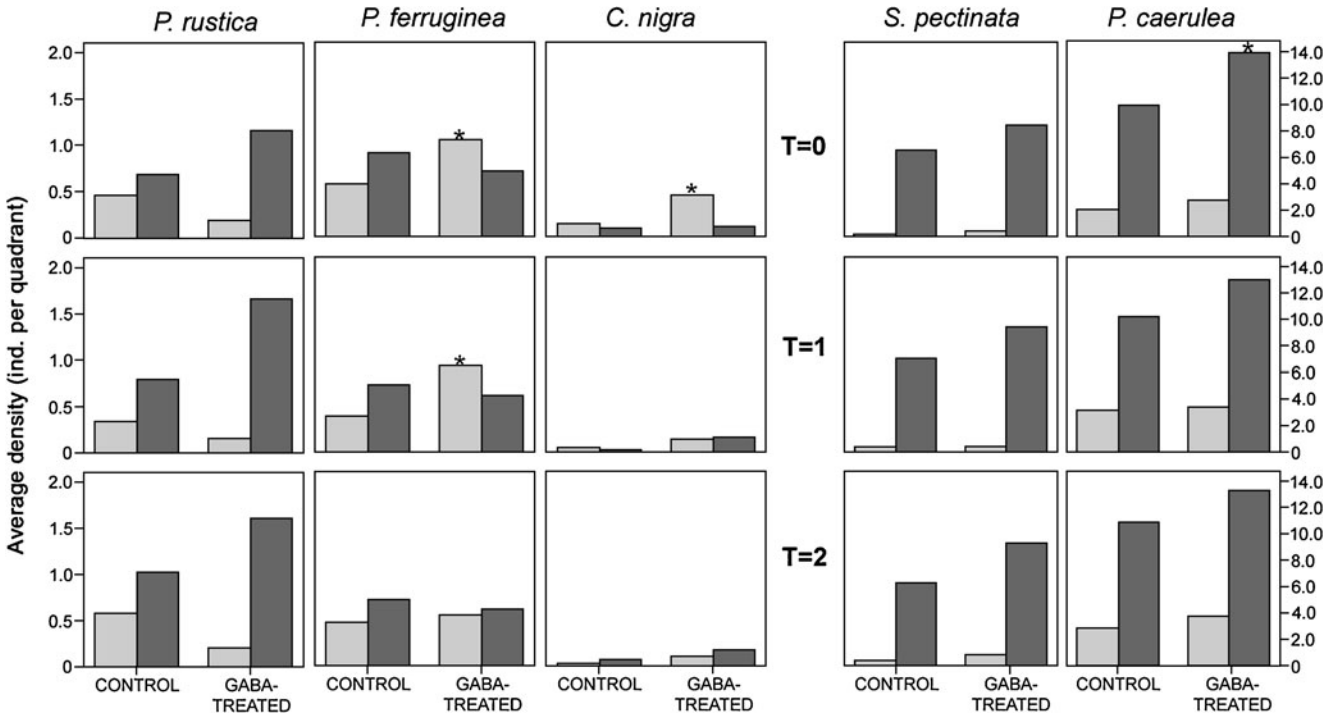


Fig. 3 Average number of individuals registered in 20×20 cm quadrant according to size, species, treatment, and sampling dates. T=0, March 2009, after the last GABA application; T=1, April 2009, one month after the last GABA application; T=2, May 2009, two months after the last GABA application. Dark bars represent adults, while grey bars represent recruits (less than 5 mm *Siphonaria pectinata*; less than 10 mm for *Patella caerulea* and *P. rustica*; less than 20 mm for *P. ferruginea* and *Cymbula nigra*). *$P<0.05$

to T=2 ($F=4.595$, $P=0.033$). This indicated that even two months after the last GABA application, there still was evidence of the effects of the solution. *Patella caerulea* recruits followed the above mentioned pattern, and individuals located around treated surfaces presented greater distances to the plates than individuals around the controls. *Patella ferruginea* did not show any differences for recruits ($F=2.346$; P = n.s.) nor for adults ($F=0.179$; P = n.s.). *Cymbula nigra* did not show differences for recruits ($F=0.378$; P = n.s.) either, but surprisingly did for adults ($F=7.727$; $P=0.024$) which were found to be significantly closer to the treated plates than to the controls (refer to Table 1 for further data).

The affect of the presence of adult conspecifics on recruitment also was inferred based on the data collected for the case of the two endangered species (*P. ferruginea* and *C. nigra*). All 90 plates used in the experiment were classified into four different categories based on the composition of adults and recruits. Figure 4 shows the results for *P. ferruginea*. Control treatments showed a considerably higher number of plates with no individuals than the positive treatment. Moreover, a high number of treated plates presented only recruits, while the number of plates presenting both adults and recruits were similar in number between treatments. Additionally, 70% of the control plates that presented *P. ferruginea* recruits could be explained by the presence of adult conspecifics. For the case of *C. nigra*, the total number of individuals recorded was not enough to conduct the analysis.

The influence of substrate inclination on recruitment also was analyzed. Vertical substrates showed the lowest number of recruits, although no significant differences were found between this and the other inclinations considered (mean values: horizontal susbtrata=4.27±4.18 individuals; inclined substrates=4.50±3.92 individuals; vertical substrates=3.34±3.60 individuals) ($F=0.72$; P = n.s). Similar-

Table 1 Mean distances to GABA application points for recruits and adults found (for each species) around GABA-treated plates for each of the considered species at T=0

Species	Mean ± SD (cm) (Recruits)	Mean ± SD (cm) (Adults)
Siphonaria pectinata	5.12±3.52	5.21±3.26
Patella caerulea	5.39±3.18[a]	4.95±3.22
Patella rustica	5.57±3.23	6.54±3.64
Patella ferruginea	5.60±2.90	5.90±2.84
Cymbula nigra	4.51±3.20	3.75±2.50[b]

[a] Significantly higher than control values

[b] Significantly lower than control values

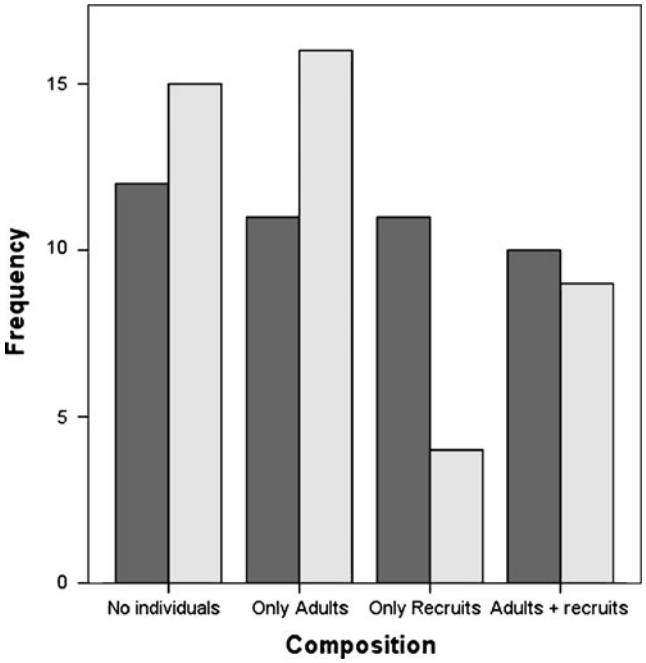


Fig. 4 Frequency of plates presenting different *Patella ferruginea* compositions based on maximum shell size. Dark bars represent GABA-treated plates, while lighter bars represent controls. (Recruits< 20 mm; Adults>20 mm)

ly, location did not apparently influence limpet recruitment either (F=1.34; P=n.s.).

Discussion

The present study presents the first results regarding the effect of GABA on patellid limpet recruitment and population dynamics. Through monitoring the colonization of experimental plates and their surrounding areas, we determined that the compound not only enhanced the recruitment processes, but also accelerated it. Surprisingly, adults were also affected by GABA, and adult *P. caerulea* immigrants were found in significantly higher numbers around the treated plates and were the first to be recorded. This is an interesting point, as many of the prosobranch limpets found on rocky shores are known to exhibit "homing behavior" (Gray and Hodgson, 1998), meaning that after foraging excursions, individuals usually return to their home scars. The fact that adults were found on plates means that these individuals have abandoned their original home scars, and established new ones on the experimental surfaces. For the case of *P. ferruginea*, this type of change has been associated with unfavorable growth conditions on the original scar (pers. obs.), because of being occupied by another individual or because low tide arrived before the individual returned to the scar (Espinosa et al., 2008). It is also reasonable to accept that recruits (especially those from *C. nigra* and *P. ferruginea*) are effectively attracted by GABA, and also present different behavior mediated by the presence of this compound.

The differences between treatments (for both adults and recruits) were usually maintained up to one month after the last GABA application. After that, the number of individuals was again similar to the ones found around controls, indicating that adults and recruits previously attracted by the solution may have changed home scar guided by some other chemical cue of natural origin. These results may suggest additionally that individuals are receptive to chemical stimuli over the entire lifespan and, in consequence, these cues may have not only a recruitment function. Moreover, it has been been shown recently that *P. ferruginea* is able to detect the presence of larger conspecifics and adjust the timing of sex change accordingly (Rivera-Ingraham 2010), meaning that chemical cues indeed play an important role during their lifetime and may also influence adults in other processes such as reproduction.

The present study also provides indirect results regarding the spatio-temporal influence of GABA on limpets. These data indicate that apart from the organisms located on the treated surfaces, individuals located in areas up to at least five centimeters around the application point also were affected. Additionally, a significant increase in the number of *S. pectinata* recruits was obtained around the treated plates four months after the last GABA application (end of July 2009). The timing of this increase could be due to the fact that the spawning period of the species occurs from March to June (Ocaña and Emson, 1999). The fact that this increase occurred around treated plates, however, could only indicate that the product had some effect on limpet communities for at least one month after the last application (when *S. pectinata* settlement probably took place).

Another important factor that should be taken into account is product concentration. The effect of GABA on patellid limpet may be a natural pattern produced as the result of the influence of a naturally-produced compound, or on the contrary, GABA may not be naturally produced by conspecifics. In the latter case, it might exert a toxic effect on the most sensitive fraction of populations, recruits. This is supported by the fact that *P. caerulea* recruits appeared with higher distances to GABA application points than to controls. However, no significantly higher mortality rates were observed on the treated plates, especially considering the fact that individuals were in direct contact with the 1 mM GABA solution as part of the normal application process. During the last decades, many studies have been conducted in order to determine the effective doses for larval settlement and tolerance to various concentrations of compounds. Regarding the latter, GABA concentrations of 0.5 μM to 10 μM have been described for

Haliotis sp. (see Morse et al., 1979a, b; Yang and Wu, 1995; Bryan and Qian, 1998; Gapasin and Polohan, 2004; Li et al., 2006; Searcy-Bernal and Anguiano-Beltrán, 2007). In all cases, however, the authors could effectively control these values as they were working in limited areas such as aquariums. Regarding toxic GABA concentrations, studies in abalone have reported values of 0.1 mM (Yang and Wu, 1995; Bryan and Qian, 1998).

Finally, we were able to determine that for the case of *P. ferruginea*, GABA seemed to produce an effect similar to that produced by the adults. Many of those control areas that showed *P. ferruginea* recruits could frequently be explained by the presence of adult conspecifics. Future studies should be conducted to determine the specific effect of adults on recruitment rates. It has been suggested previously that chemical cues may be involved in patellid limpet recruitment (Laborel-Deguen and Laborel, 1990), but this is the first evidence that directly supports this hypothesis.

Conservational Implications for Endangered Species Taking into account the results and observations of the present study, new considerations can be made regarding the proposal of obtaining *P. ferruginea* recruits through the use of artificial plates that could be later translocated for conservational purposes (Spanish National Strategy for the Conservation of *P. ferruginea*) (MMAMRM, 2008). We suggest the use of limestone plates similar to the ones described here. These plates should present a medium to high heterogeneity surface (surface rugosity coefficient >1.15, measured as in Wright, 1983), as the presence of microstructures have been proven to favor settlement and recruitment as well as serve as refuge and increase larval survival rates (Bulleri, 2005; Moschella et al., 2005; Rivera-Ingraham, 2010). The use of large surfaces should be avoided, as they can be torn easily apart by wave action, as has occurred in a recent study developed by Espinosa et al. (unpublished results).

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Ontogeny of Tetrodotoxin Levels in Blue-ringed Octopuses: Maternal Investment and Apparent Independent Production in Offspring of *Hapalochlaena lunulata*

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Abstract Many organisms provision offspring with antipredator chemicals. Adult blue-ringed octopuses (*Hapalochlaena* spp.) harbor tetrodotoxin (TTX), which may be produced by symbiotic bacteria. Regardless of the ultimate source, we find that females invest TTX into offspring and offspring TTX levels are significantly correlated with female TTX levels. Because diversion of TTX to offspring begins during the earliest stages of egg formation, when females are still actively foraging and looking for mates, females may face an evolutionary tradeoff between provisioning larger stores of TTX in eggs and retaining that TTX for their own defense and offense (venom). Given that total TTX levels appear to increase during development and that female TTX levels correlate with those of offspring, investment may be an active adaptive process. Even after eggs have been laid, TTX levels continue to increase, suggesting that offspring or their symbionts begin producing TTX independently. The maternal investment of TTX in offspring of *Hapalochlaena* spp. represents a rare examination of chemical defenses, excepting ink, in cephalopods.

Key Words Tetrodotoxin · Blue-ringed octopus · *Hapalochlaena lunulata* · *Hapalochlaena fasciata* · Maternal investment · Egg · Paralarva · Ontogeny

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Introduction

Many chemically defended organisms endow their offspring with their own toxic protection. Examples of plant seeds invested with secondary metabolites are ubiquitous (reviewed by Janzen, 1978). Insects, through physiological resistance to plant toxins, have repeatedly co-opted plant defensive compounds for their own and their offsprings' defense. For example, moths of the genus *Utetheisa* provision their eggs with pyrollizidine alkaloids that were accumulated from their legume host, and these alkaloids protect clutches from predators and parasitoids (Eisner and Meinwald, 1995; Eisner et al., 2000; Bezzerides et al., 2004). Although vertebrate examples are fewer, amphibians are known for defensive compounds produced in granular glands (reviewed by Toledo and Jared, 1995), and many amphibians provision their eggs with these defensive compounds (e.g., Fuhrman et al., 1969). Rough skinned newts (*Taricha*) endow eggs with tetrodotoxin (TTX; e.g., Hanifin et al., 2003), and toads (*Rhinella*) invest eggs with bufadienolides (e.g., Akizawa et al., 1994). An Asian snake (*Rhabdophis tirgrinus*) even sequesters these bufadienolides from its bufonid prey (*Bufo*), and transfers the usurped chemical defenses to its own offspring (Hutchinson et al., 2008).

Many marine vertebrate and invertebrate eggs and larvae also are invested with a chemical defense from a toxic

parent. For example, at least two ascidian species (sea squirts or tunicates) produce such deterrents as the bipyrrole alkaloid tambjamine C and several didemnin cyclic peptides that render both adults and larvae distasteful to predators (Lindquist et al., 1992). Puffer fish eggs often contain tetrodotoxin (TTX), as do adults (Kao, 1966; Fuhrman et al., 1969). Examples of maternal provisioning also exist in both terrestrial and marine gastropods. Miriamin is an antipredator diterpene invested in the eggs of a slug (*Arion* sp.; Schroeder et al., 1999), and the nudibranch (*Hexabranchus sanguineus*) redirects antipredator macrolides sequestered from its sponge diet into egg ribbons (Pawlik et al., 1988). Clearly, maternal provisioning of offspring with defensive compounds is prevalent across the spectrum of life on earth, whether the toxin is produced endogenously or obtained exogenously by the donating parent.

However, provisioning offspring with chemical defenses may be costly. Metabolic costs of defense compete with growth and reproduction, for example, in aspens and wild parsnip, respectively (Zangerl and Berenbaum, 1997; Häikiö et al., 2009). Additionally, autotoxicity from defensive compounds in young embryos may inhibit growth (e.g., Orians and Janzen, 1974). These costs likely explain, in part, why all chemically defended adults do not invest offspring with defensive compounds (e.g., Tarjuelo et al., 2002).

When present, the chemical defenses in eggs and offspring often coincide with adults bearing the same toxin (Lindquist et al., 1992). Adult blue-ring octopuses are well-known for possessing the potent neurotoxin, TTX, which has been identified in 3 species of blue-ringed octopus, *Hapalochlaena fasciata* (e.g., Sheumack et al., 1978; Williams and Caldwell, 2009), *H. maculosa* (e.g., Yotsu-Yamashita et al., 2007), and *H. lunulata* (e.g., Williams and Caldwell, 2009). Based on symptoms of human envenomation (e.g., McMichael, 1964), other species of *Hapalochlaena* also appear to contain TTX. Sheumack et al. (1984) identified TTX in the eggs of one of these species (probably *H. fasciata* based on the authors' locality), but quantity, variation, and ontogeny of the TTX were not described. Although the ultimate origin of the TTX present in octopuses is unclear, TTX appears to be produced by bacteria in the posterior salivary gland (PSG) of *H.* cf. *lunulata* from the Philippines (Hwang et al., 1989).

Regardless of the source in adults, TTX also may serve as a powerful defense (e.g., Mosher et al., 1964; Kawasaki et al., 1973) against predation for eggs and young blue-ringed octopuses. If TTX is defensive, investment by *H. lunulata* and *H. fasciata* in offspring and the ontogeny of TTX production in *H. lunulata* would add to the sparse knowledge of chemical defenses in cephalopods. One unusual octopod gathers Portuguese Man-o-war tentacles and presents the cnemotocyst armory to potential predators (Jones, 1963). However, the most common and well-studied form of chemical defenses in cephalopods is ink (reviewed by Hanlon and Messenger, 1996; Derby, 2007). Investigations of other chemical defenses, and the maternal investment thereof, are few.

Other adult cephalopods, such as the flamboyant cuttlefish (*Metasepia pfefferi*) and the striped pyjama squid (*Sepiolodia lineolata*), are known to be toxic (Norman, M., pers. comm.). Additionally, an unnamed arm dropper octopus (*Abdopus* species 5) contains saxitoxin, which is functionally similar to TTX (Robertson et al., 2004). Any of these cephalopods also may invest chemical defenses into their offspring.

Although the presence of TTX has been identified in the eggs of *H. fasciata* (Sheumack et al., 1984), the ontogeny of TTX quantities has not been examined in this or other *Hapalochlaena* species. *Hapalochlaena lunulata* lay large clutches (~100–300 eggs), and clutches hatch diurnally and synchronously (Overath and von Boletzky, 1974; pers. obs.)—three conditions that correlate with toxicity in other pelagic larvae (Lindquist and Hay, 1996). Here, we determined whether female *H. lunulata* provision their eggs with TTX and whether these stores persist in paralarvae at hatching. We investigated TTX levels of adult females, paralarvae, and eggs at multiple stages of development to ascertain the relationship between maternal and offspring TTX levels, and to evaluate the ontogeny of TTX levels through hatching.

Methods and Materials

Sampling We obtained 18 female, 42 male, and 12 sex unknown adult and sub-adult *Hapalochlaena lunulata,* shipped from Bali, Indonesia, through the tropical aquarium industry between June 2004 and November 2006. Octopuses were wild-caught at our request and according to the importer were in transit no more than 1 wk. We also obtained 5 male and 2 female adults of *Octopus cf. mercatorus* via the pet trade from Florida; these small octopuses occupy a habitat similar to that of *H. lunulata* and provide a non-TTX bearing control. Octopuses were housed in 38 L aquaria. Artificial seawater was mixed at 34 ppt (Aqua Craft, Inc.). Every third day octopuses were fed live grass shrimp, ghost shrimp, snails, brine shrimp, or thawed prawn or fish pieces as availability permitted. Males were introduced into home aquaria of the females for mating (see Cheng and Caldwell, 2000 for a detailed analysis of mating). Females that deposited eggs were offered food approximately twice weekly. However, these semelparous octopuses often forgo feeding during brooding and expire soon after eggs are hatched (Tranter and Augustine, 1973; Overath and von Boletzky, 1974), and this pattern was consistent with the

H. lunulata and *O. cf. mercatorus* we observed. Only one female (*H. lunulata*) continued to feed after oviposition, albeit at a reduced frequency and volume as compared to her pre-oviposition meals.

Opportunistically, we were able to examine two female *H. fasciata*—one with hatchlings and one sexually immature individual. Unlike *H. lunulata,* which produce pelagic paralarvae (Overath and von Boletzky, 1974), *H. fasciata* produce benthic hatchlings (Tranter and Augustine, 1973). The adult that produced hatchlings was collected with eggs by hand near the Manly Ferry Station in Manly Cove, New South Wales, Australia, in February 2006. Hatchlings emerged from the eggs within 4 hr after collection, at which time the female and hatchlings were frozen at −20°C. In April 2006, this female was dissected, and her tissues and individual hatchlings were desiccated for 48 hr using Dririte©. An additional female was collected in April 2006 near the Manly boat harbor and transported live to the United States. Upon our return to the lab, both fresh and desiccated samples were frozen at −80°C before analysis for TTX.

Nine female *H. lunulata* and their paralarvae or eggs (collected before and after oviposition) were sampled at various stages of development; we divided the range of samples into eight stages (Table 1). For detailed reviews of cephalopod development, see von Boletzky (1989, 2003). Tetrodotoxin content in the posterior salivary glands (PSG) was used as a proxy for total female TTX levels because these two quantities are highly correlated and PSG is more easily assayed (Williams and Caldwell, 2009). Toxicity assays are destructive, thus female samples were collected at the reproductive stage indicated by eggs/paralarvae. Previously, we found TTX in oviducal glands of *H. fasciata* (Williams and Caldwell, 2009)*;* thus, three oviducal glands of *H. lunulata* and one of *H. fasciata* also were sampled.

Tetrodotoxin Extraction and Quantification We extracted TTX by grinding the sample in 100 µl (for eggs and oviducal glands) or 200–400 µl (for PSG) of 0.05 N acetic acid with a 1 ml Dounce glass homogenizer (Wheaton, USA). Extracts were heated to 100°C in boiling water for 5 min to denature protein contaminants, cooled in an ice bath, and centrifuged at 13,000 rpm for 15 min. We then filtered the supernatant through 5,000 nominal molecular weight limit 0.5 ml Ultrafree-MC Millipore tubes by centrifugation for 20 min at 13,000 rpm. Filtrates were stored at −80°C until quantification.

We quantified TTX in sample extracts using reverse phase high performance liquid chromatography (HPLC) by modifying the procedures of Yotsu et al. (1989), and Hanifin et al. (1999). Twenty µl aliquots of samples were eluted with an isocratic gradient of 1.0% by volume acetonitrile, 1.28% by weight heptafluorobutyric acid, and 0.29% by volume acetic acid. The mobile phase was adjusted to pH 5.0 with 50% NH_4OH. We separated analytes with a Synergi 4 µ Hydro-RP 80A C18 column (250×4.6 mm, Phenomenex, USA). A Beckman Coulter System Gold 126 Solvent Module provided a primary flow rate of 0.5 ml per min for the mobile phase and a secondary flow rate of 1.0 ml per min of 5 N sodium hydroxide for mixing post-column. A Pickering CRX 400 reactor (1 ml reaction volume) then heated elutants to 115°C after mixing to break down TTX and any possible analogs. A water jacket cooled the derivatization products before detection by a Jasco FP-1512 fluorescence detector with excitation wavelength set at 365 nm and emission wavelength at 510 nm. Beckman System Gold Software recorded the chromatographs and calculated peak areas. Tetrodotoxin peaks in tissue samples were identified and quantified by comparison with a standard (Sigma) of 1.0 µg/ml TTX in 0.10 N acetic acid. Tissues and offspring of *H. lunulata* and *H. fasciata* were compared with those of *O. cf. mercatorus* as a control. We used a peak skimming formula to conservatively calculate peak areas where the TTX peak eluted on top of a broad band.

Table 1 Descriptions of developmental stages assigned to eggs and paralarvae

Dev. Stage	Description
1	eggs largely undifferentiated in organic matrix
2	eggs differentiated, but still in organic matrix
3	eggs formed in ovary, chorion deposited by follicular syncytium of the ovary (Boyle and Chevis, 1992)
4	eggs deposited
5	1 wk after eggs deposited
6	2 wk after eggs deposited, eyespots have formed
7	3 wk after eggs deposited, chromatophores functional, eyes developed, and embryos have begun turning in preparation for hatching
8	paralarvae hatched (~ 4 wk after eggs deposited)

Statistical Analyses We used multiple regression analyses to evaluate: 1) the influence of female TTX levels and developmental stage on mean TTX levels of egg(s) or paralarva(e); and 2) the influence of female size and developmental stage of eggs on female TTX levels. For these two analyses, variables were transformed by the equation log (X+0.1) to better approximate a normal distribution (as evaluated by Shapiro-Wilk tests). Where replicates were available (indicated by sample size and SE in Table 3), we used the mean TTX levels of offspring to avoid pseudoreplication in the multiple regression. In addition, only stage 6 offspring of female #28 were used in this analysis to avoid psuedoreplication; this was the only female we were able to sample at multiple time points.

We used mantle length as an indicator of female size because a significant portion of the female's body mass is likely lost during egg deposition, whereas mantle length will remain more constant. Tetrodotoxin levels were measured as μg TTX/g tissue. We also were able to track toxicity within one clutch over development (female #28). With these data we analyzed how egg and paralarvae toxicity changed with developmental stage by using Spearman's Rank Correlation (ρ). Analyses were performed in JMP 7© 2007.

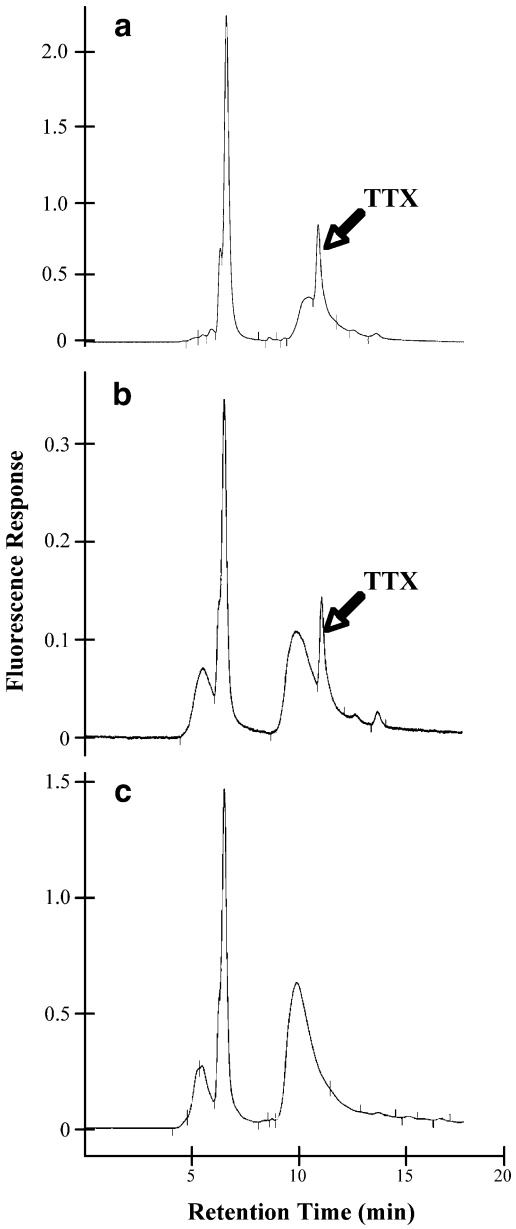


Fig. 1 Chromatograph illustrating the presence of tetrodotoxin (TTX) in tissues of *Hapalochlaena lunulata*: **a** Profile of the posterior salivary gland of an adult female #27 **b** Profile of a whole paralarva from female # 28 within 24 hr of hatching (stage 8 Table 1) **c** Profile of the control hatchling (*Octopus cf. mercatorus*) showing an absence of TTX

Table 2 Tetrodotoxin (TTX) levels in *Hapalochlaena fasciata*

	Female 4	Female 5
μg TTX in PSG	29.10	0.06
ng TTX per ovary	420	NE
ng TTX per oviducal gland	210	NE
ng TTX per hatchling (N=3)	NE	90, 150, 180

Results

Two clutches of *H. lunulata* (females # 4 and 28) and two clutches of *O. cf. mercatorus* developed successfully to hatching. Two additional *H. lunulata* females deposited eggs that did not hatch; one set of eggs did not develop (female #13) and one set began development but ceased after the death of the brooding female (#27). Tetrodotoxin was detectable in many, but not all of the tissues sampled (Fig. 1). Tetrodotoxin was the main and prominent analog, with traces of the presumed equilibrium products 4-epi TTX and 4,9 anhydro TTX (e.g., Goto et al., 1965; Mosher, 1986); these are represented (their identities are inferred only as they were not compared with authentic standards, which were unavailable) by the two peaks following that of TTX with retention times of ~13 and 14 min, respectively (Fig. 1). The proportion of these equilibrium products did not appear to change over time, nor were any other analogs of TTX apparent at any timepoint. No traces of TTX or its analogs were present in adult tissues or hatchlings of control octopuses (*O. cf. mercatorus*). No TTX was apparent in oviducal glands of the 3 *H. lunulata* tested, but TTX was present in the oviducal gland in the single *H. fasciata* for which this tissue was tested (Table 2). The benthic young of the second of the two female *H. fasciata* contained TTX (Table 2). Considerable variation in TTX levels was found between and within clutches and between females of *H. lunulata* (Table 3). Even so, several trends were readily discernable.

Egg/parlarva TTX levels (μg TTX/g tissue) were positively correlated with female TTX levels (μg TTX/g tissue) after accounting for the effect of developmental stage (N=9, df=1, F=7.98, P=0.030). Egg/paralarva TTX levels showed an increasing, but non-statistically significant, trend with respect to developmental stage (N=9, df=1, F=5.60, P=0.056). The within-clutch analysis of development post-oviposition by Spearman's Rank Correlation, however, revealed that egg–paralarva toxicity (ng TTX/egg or hatchling) did increase from stage 6 to 8 (N=9, ρ= 0.8808, P=0.002; Fig. 2). Because females cease feeding

Table 3 Tetrodotoxin (TTX) levels in *Hapalochlaena lunulata* and their offspring*

Female ID	Mean μg TTX per g paralarvae or egg (± SE)	*N*	Dev. Stage	Female Mantle Length (mm)	μg TTX per g PSG of Female	Total TTX in PSG of Female (μg)
7	0	~8*	1	23	0.21	0.01
9	3.64	~12*	1	32	114.34	12.12
11	3.12	~24*	1	35	55.45	4.88
15	15.84 (1.18)	2	2	28	65.4	7.32
5	0	1	3	22	1.33	0.04
13	0.24 (0.21)	3	4	15	0.08	0
27	17.05 (6.91)	3	7	24	37.14	1.56
4	6.11 (4.46)	3	8	23	0.35	0.01
28	5.93 (2.44)	3	6	18	BDL	BDL
28	9.01 (4.84)	3	7	18	BDL	BDL
28	16.14 (5.88)	3	8	18	BDL	BDL

N=number of samples

BDL Below detectable level

*Female identification numbers correspond to those in Williams and Caldwell (2009) and females # 27 and 28 correspond to collector # BLW 198 and 131, respectively

**Individual eggs were unformed at developmental stage 1 and sample size represents the best estimate for the number of eggs that were forming; these samples were pooled into a single toxicological analysis and included the organic matrix surrounding the developing egg mass

during brooding (Tranter and Augustine, 1973; Overath and von Boletzky, 1974) and given that egg/parlarva TTX levels increase as development progresses, one might expect to see female TTX levels decrease concordantly. However, this was not the case; female TTX levels (μg TTX/g tissue) were not related to offspring developmental stage (N=9, df=1, F=0.245, P=0.636). Female TTX levels were, however, positively correlated with female size (N=9, df=1, F=11.96, P=0.014).

Discussion

Both *H. lunulata* and *H. fasciata* appear to invest TTX into their offspring. Tetrodotoxin was present in the ovary of both species at the earliest stage of development before individual eggs had differentiated and would be capable of producing their own TTX or individually supporting bacterial symbionts. Thus, we conclude that females influence the distribution of TTX stores and possibly the distribution or provisioning of any TTX-producing symbionts. This is further supported by the positive relationship between egg/parlarva TTX levels and female TTX levels in *H. lunulata,* which may suggest either passive diffusion into eggs or female control over this chemical investment. TTX was invested in eggs even as they were aggregating from the organic matrix of the ovary (stage 1 in this study; Table 1) when females are still actively foraging and accumulating resources before mating and reproduction. At this time, the potential for an adaptive tradeoff is greatest between investment in offspring and self-defense or use of TTX as venom for prey capture. In contrast to previous work on *H. lunulata* (Williams and Caldwell, 2009), we found female size positively correlated with TTX levels.

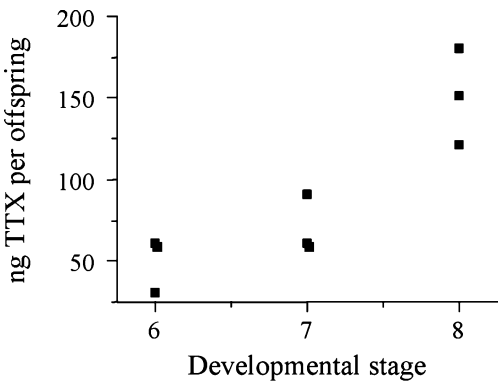


Fig. 2 Increase of tetrodotoxin (TTX) in 9 embryos from one clutch (female #28) of *Hapalochlaena lunulata* from 2 weeks after eggs were laid (stage 6) through hatching (stage 8; N=9, ρ=0.8808, P=0.002)

Eggs of some species provisioned with chemical defenses, such as those of the western toad (*Anaxyrus boreus*), lose toxicity during development (e.g., Benard and Fordyce, 2003). Time held in the lab may result in decreased TTX levels in *H. lunulata* if bacterial symbionts produce the TTX and are negatively affected by the captive environment. However, we found that TTX stores in *H. lunulata* embryos increase throughout egg and paralarval development, even after oviposition (Fig. 2). Females of *H. lunulata* and *H. fasciata* brood eggs until hatching (Tranter and Augustine, 1973; Overath and von Boletzky, 1974);

thus, continual investment of TTX into eggs by females theoretically is possible. If additional TTX is provided by females after oviposition, we might expect TTX levels in females to drop over development; however, our data revealed no such trend. Additionally, to our knowledge, there is no evidence of transfer of maternal provisions, such as nutrients or chemical defenses, from maternal octopuses across the chorion to embryos after oviposition. Because TTX is water soluble, a carrier would be required to transfer TTX to the eggs to prevent diffusion into the seawater. Seemingly, investing TTX into eggs before the deposition of the egg coat and cement, which serves as an environmental barrier, rather than continuing provision after oviposition would be a more effective method of transfer. While females could coat the eggs with external chemical defenses, we noted no external coating during egg collection. Further, TTX levels in freshly hatched paralarvae nearly triple after oviposition (Fig. 2), suggesting that the increase in TTX is not due simply to a coating on the eggs, which is discarded during hatching. Thus, although continued female investment of TTX after oviposition should be ruled out in future work, we suspect that embryos or the their symbionts begin production of TTX independently of female investment after oviposition.

Hwang et al. (1989) reported TTX-producing bacteria from the posterior salivary glands (PSG) of *H. cf. lunulata* from Cebu Island, the Philippines. Thus, the possibility exists that females inoculate developing embryos with TTX-producing bacteria. Female squid harbor symbiotic bacteria in accessory nidamental glands (ANG) and pass these bacteria onto the egg coat of their young (e.g., Biggs and Epel, 1991). However, the ANG that houses these symbionts is not fully developed in hatchling squid, and their ANG is colonized much later, from bacteria in the surrounding seawater rather than from maternal bacteria (Kaufman et al., 1998). In cephalopods, the nidamental glands and oviducal glands both deposit substances on the egg coat (chorion) before oviposition (reviewed in von Boletzky, 2003). Immediately prior to oviposition in octopuses, the oviducal gland coats the eggs with two secretions that harden into cement upon contact with sea water (Froesch and Marthy, 1975). Here, the 3 *H. lunulata* tested showed no evidence of TTX in their oviducal glands; however, 2 *H. fasciata* possessed TTX in these organs (this study; Williams and Caldwell, 2009). Whether symbiotic bacteria reside in the oviducal gland in addition to PSG remains untested. We verified that females invest eggs with TTX by demonstrating the presence of TTX in the ovary before individual eggs were formed and by the correlation between female and offspring TTX levels—a relationship that would not be present if females simply inoculated offspring with bacterial symbionts (Piel, 2004). However, we were unable to ascertain whether these females also provide bacteria to developing embryos.

Just as we found that TTX increases in octopus embryos after laying, TTX increases in puffer fish embryos (Matsumura, 1998). Matsumura (1998) takes this as an indication of endogenous production of TTX in the puffer fish rather than vertical transmission of symbiotic bacteria. While Matsui et al. (1982) found captive puffer fish (*Takifugu niphobles*) lacked TTX, Matsumura (1996) found that captive puffer fish (*Takifugu rubripes*) did possess low levels of TTX. Saxitoxin is structurally similar to TTX (Kao, 1966), and also was previously thought to be produced by symbiotic bacteria (reviewed by Piel, 2004). Martins et al. (2003) and Matsumura (1995, 2001) note that techniques for identifying saxitoxin and TTX production in bacteria, respectively, are not specific enough, and the media used to grow the purported symbionts actually yield a false positive identification of the toxins. These are the same techniques used by Hwang et al. (1989) to identify TTX production in bacteria of *Hapalochlaena*; thus, their conclusions have been called into question and need additional verification before the presence of bacterial symbionts in the octopuses can be accepted.

Regardless of the origin of TTX in eggs and young octopuses, the investment of TTX in young may be costly. The multi-ring structure of this small molecule (Goto et al., 1965; Tsuda et al., 1964; Woodward, 1964), the difficulty in identifying a natural biosynthetic pathway (Shimizu and Kobayashi, 1983; Ohyabu et al., 2003), and the stereospecificity of TTX (Furusaki et al., 1970) all suggest that it is not a byproduct of another pathway and may be metabolically expensive to produce. An octopus (or its symbiotic bacteria) can produce more than 0.4 mg of TTX (Williams and Caldwell, 2009), representing a potentially substantial investment. If symbionts produce the TTX, the cost of maintaining symbiotic colonies may be sizeable, as it is in plant-mycorrhizae and nematode-bacteria interactions (e.g., Cameron et al., 2008; Emelianoff et al., 2008). Although dietary bioaccumulation of TTX occurs in some organisms (e.g., pufferfish; Kono et al., 2008) and may represent a lower-cost source of the toxin, dietary accumulation of TTX by these octopuses is unlikely to be the primary source because egg/parlarva TTX levels increase after oviposition but before feeding (Fig. 2), and maternal TTX levels do not decrease concordantly. If TTX production is costly, this increase suggests adaptive benefits of the toxin for the young octopuses.

Tetrodotoxin may serve adaptively as a chemical defense or offense in blue-ringed octopus eggs and paralarvae or hatchlings. Many puffer fish have TTX in their eggs (e.g., Kao, 1966; Fuhrman et al., 1969), and Gladstone (1987) found that eggs and larvae of Sharpnose pufferfish (*Canthegaster valentini*) are distasteful to two other species

of reef fish. Gladstone (1987) speculates that TTX conferred the distastefulness but had no empirical evidence that eggs contained TTX or that TTX repelled the predators tested. The antipredator efficacy of TTX has only been empirically demonstrated in one species of newt (*Taricha granulosa*; e.g., Williams et al., 2010). In the genus *Hapalochlaena* the function of adult TTX is assumed to be both venom and defensive compound. Whether TTX provides an antipredator defense or represents developing venom for paralarvae or hatchlings of blue-ring octopuses is currently unknown.

In summary, females of *H. lunulata* and *H. fasciata* invest TTX into their offspring. Counter to predictions, embryos increase in TTX levels even after oviposition; however, female TTX levels do not decrease concordantly. This may indicate that embryos or their bacterial symbionts begin independent production of TTX before hatching. Whether females inoculate eggs with TTX-producing bacteria is unknown, as is the ecological function of TTX in the eggs and paralarvae or hatchlings. Work is necessary to determine the ultimate origin of TTX in blue-ringed octopuses (*Hapalochlaena* spp.) and the functional ecology associated with this potent neurotoxin in this group.

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Volatile Emissions from *Alnus glutionosa* Induced by Herbivory are Quantitatively Related to the Extent of Damage

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Abstract Plant volatile organic compounds (VOCs) elicited in response to herbivory serve as cues for parasitic and predatory insects. Knowledge about quantitative relationships between the extent of herbivore-induced damage and the quantities of VOCs released is scarce. We studied the kinetics of VOC-emissions from foliage of the deciduous tree *Alnus glutinosa* induced by feeding activity of larvae of the geometrid moth *Cabera pusaria.* Quantitative relationships between the intensity of stress and strength of plant response were determined. Intensity of biotic stress was characterized by herbivore numbers (0–8 larvae) and by the amount of leaf area eaten. The strength of plant response was characterized by monitoring (i) changes in photosynthesis, (ii) leaf ultrastructure, and (iii) plant volatiles. Net assimilation rate displayed compensatory responses in herbivore-damaged leaves compared with control leaves. This compensatory response was associated with an overall increase in chloroplast size. Feeding-induced emissions of products of the lipoxygenase pathway (LOX products; (*E*)-2-hexenal, (*Z*)-3-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate) peaked at day 1 after larval feeding started, followed by an increase of emissions of ubiquitous monoterpenes peaking on days 2 and 3. The emission of the monoterpene (*E*)-β-ocimene and of the nerolidol-derived homoterpene 4,8-dimethyl-nona-1,3,7-triene (DMNT) peaked on day 3. Furthermore, the emission kinetics of the sesquiterpene (*E,E*)-α-farnesene tended to be biphasic with peaks on days 2 and 4 after start of larval feeding. Emission rates of the induced LOX products, of (*E*)-β-ocimene and (*E,E*)-α-farnesene were positively correlated with the number of larvae feeding. In contrast, the emission of DMNT was independent of the number of feeders. These data show quantitative relationships between the strength of herbivory and the emissions of LOX products and most of the terpenoids elicited in response to feeding. Thus, herbivory-elicited LOX products and terpenoid emissions may convey both quantitative and qualitative signals to antagonists of the herbivores. In contrast, our data suggest that the feeding-induced homoterpene DMNT conveys the information "presence of herbivores" rather than information about the quantities of herbivores to predators and parasitoids.

Key Words Biotic stress · Green leaf volatiles · Leaf ultrastructure · Photosynthesis rate · Volatile organic compounds

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Introduction

Plants have developed a variety of defense strategies that cope with insect attacks. Direct defenses act directly against the herbivores, whereas indirect plant defenses may act by release of plant volatile organic compounds (VOCs) that attract natural enemies of the herbivores (see for reviews Gershenzon and Dudareva, 2007; Poelman et al., 2008; Dicke, 2009). Quantitative and qualitative herbivore-induced changes in VOC emissions have attracted special attention (Holopainen, 2004; Arimura et al., 2005; Brilli et

al., 2009; Dicke, 2009; Kännaste et al., 2009; Wu and Baldwin, 2009; Schaub et al., 2010).

The first, almost instantaneous response of plants to wounding is the emission of volatile products of the lipoxygenase pathway, i.e., LOX products (various C6-aldehydes, -alcohols, and -esters, also called green leaf volatiles; Pinto et al., 2007; Gosset et al., 2009). Lipoxygenase (LOX) pathway products are induced in a variety of plant species during different stress conditions (e.g., Heiden et al., 2003; Copolovici and Niinemets, 2010) in a process where free octadecanoid fatty acids are released from plant membranes by phospholipases. As further responses to continued herbivory, emissions of LOX volatiles are enhanced and a wide range of other herbivore-induced VOCs is released: e.g., terpenes produced via the mevalonic acid (MVA) pathway and/or the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway or methyl salicylate biosynthesized via the shikimic acid pathway (Holopainen, 2004; Dudareva et al., 2006).

Although extensive evidence for the induction of plant volatiles by herbivory is available, studies of herbivore-induced plant VOCs often provide only information of the spectrum of compounds emitted, but no quantitative data (De Boer et al., 2004; Kännaste et al., 2008, 2009). Even in quantitative analyses that have determined the amount of compounds emitted by damaged plants (Dicke et al., 1999; Mumm et al., 2003; Schuman et al., 2009), quantitative relationships between the amounts of compounds emitted and degree of damage or the number of insects feeding rarely have been reported (Gouinguene et al., 2003). However, the strength of the signal may significantly alter the degree of attraction of the parasitoids and predators of herbivores (De Boer and Dicke, 2004; Chen and Fadamiro, 2007; Bruinsma et al., 2009). Thus, quantitative relationships between plant VOC emissions and degree of herbivory damage are of key importance for understanding multitrophic plant—insect interactions. Stress strength vs. emission response relationships have been studied for a few abiotic stresses (Beauchamp et al., 2005; Niinemets, 2010). These studies provide evidence that plant VOC emissions are quantitatively associated with stress strength. Such quantitative relationships are needed to develop large-scale models of VOC release in response to biotic stresses (Arneth and Niinemets, 2010).

In the current study, we tested the hypothesis that plant VOC emissions are quantitatively associated with the degree of damage by herbivores. We used *Alnus glutinosa*, an early successional alder species, and larvae of the geometrid moth *Cabera pusaria* for our analysis. The plant species we studied is a temperate deciduous tree species that is wide-spread in Europe and is colonizing habitats along streambanks and wet forests. Due to the capacity of nitrogen-fixation, protein-rich foliage of *Alnus spp*. is under strong herbivory pressure in natural environments. So far, only a few studies have investigated VOCs induced by herbivore attack in *Alnus* spp. (e.g., Dolch and Tscharntke, 2000; Tscharntke et al., 2001; Giertych et al., 2006; Blande et al., 2010). Larvae of *C. pusaria* are common defoliators of deciduous trees throughout the Palearctic region; the oligophagous larvae feed solitarily and externally in the foliage of deciduous trees late in the season. Herbivorous larvae, such as geometrids, cause considerable damage to deciduous trees, ranging from the consumption of a notable percentage of total leaf area (Marquis and Whelan, 1994; Martel and Mauffette, 1997) to complete defoliation in extreme cases (Wesolowski and Rowinski, 2006; van Asch and Visser, 2007).

We analyzed the kinetics of VOC emission in *A. glutinosa* leaves during and after grazing by *C. pusaria* larvae, and we determined quantitative relationships between the intensity of stress (leaf damage and herbivore numbers) and strength of plant response (photosynthesis and emissions of the lipoxygenase pathway products and terpenes).

Methods and Materials

Plant Material Alnus glutinosa plants were grown from seeds (seed source: Oudewater, The Netherlands, 52° 01′N, 4° 52′E). After germination, seedlings were grown in 3 L clay pots filled with a 1:1 mixture of quartz sand and commercial potting soil (Biolan Oy, Finland) under light intensity of 200 μmol m^{-2} s^{-1} (HPI-T Plus 400 W metal halide lamps, Philips) for a 12 h light period and day/night temperatures of 24/18°C. Plants were watered daily and fertilized once per month with slow release NPK (3-1-2 ratio) fertilizer containing microelements. In all experiments, we used similar-sized 1-yr-old seedlings with 15–20 leaves.

Insects Larvae of *Cabera pusaria* were collected from a deciduous-conifer mixed forest (58°23′N, 26°40′E) in the vicinity of Tartu, Estonia, and reared in the laboratory for 15 d in 200 ml containers at varying temperatures to synchronize the larval stages. Before the experiment, larvae were supplied with fresh alder leaves that were changed on alternate days. We used larvae in the beginning of their final instar for the experiments; at this age they have gained about 350 to 400 mg in body mass.

Experimental Setup Whole plants were placed in a dynamic headspace sampling cuvette system consisting of four 3 L glass chambers described in detail by Toome et al. (2010). In this system, above-ground plant parts were hermetically sealed in individual cuvettes, while the root system with

potting medium stayed outside. The air flow through each chamber was 0.3 $L min^{-1}$, and a fan (Sunon Group, Bejing, China) was installed in the chamber resulting in high turbulence. Assuming fully turbulent conditions and first-order exponential decay kinetics, the half-time of the chamber air corresponding to this chamber size and the flow rate was 6.9 min. (Li-Cor Inc., 2001; Niinemets, 2011). During the experiment, light was provided by the HPI-T Plus 400 W metal halide lamps for a 12 h photoperiod. Light intensity at the leaf surface was 200 $\mu mol\ m^{-2}\ s^{-1}$. After hermetic installation of the plants (day -1), they were left untreated for 24 h for stabilization. By the end of the stabilization period (day 0), treatment was started by placing 2, 4, or 8 larvae of *C. pusaria* on individual plants, while one plant was left untreated as control. After introduction of the larvae, plants were kept in the system in the dynamic air flow for one more hour before VOC sampling. Larvae were allowed to feed from the leaves for 4 d. After 4 d, they were removed, and the plants were maintained in the chambers for a further day for the collection of VOCs.

Morphological and Chemical Measurements On the 5th day, all leaves were harvested and scanned at 300 dpi. Projected leaf area was determined from the scanned images by custom made software. Leaf fresh mass was then determined immediately, and leaf dry mass was estimated after drying the leaves for 48 h at 70°C. Nitrogen content was determined by Vario Max CNS (Elementar Analyse System GmbH, Hanau, Germany).

The experiment was conducted on plants with fully mature leaves, and no leaf area expansion was observed throughout the experiment in either the control or insect-treated plants. Leaf area eaten by larvae was calculated as the difference between leaf area before the start of the experiment and the leaf area after all measurements.

Anatomical Measurements Samples for anatomical measurements were collected at the end of the experiment. From the middle part of a fully developed leaf, 1×1 mm pieces were cut between the main veins. In the damaged plants, all leaves were partly damaged, and leaves with ca. 10% damage were sampled, while intact leaves were sampled in the control plants. Leaf material was fixed by infiltrating with 2% glutaric aldehyde in 0.2 M phosphate buffer (pH 7.2) in a syringe under negative pressure. After fixation, leaf material was dehydrated in ethanol and finally embedded into Epoxy Embedding Medium (Sigma-Aldrich Chemie, Steinheim, Germany) as described by Bozzola and Russell (1992). After embedding and curing in a ventilated oven at 60°C for 48 h, semi-thin (0.5–0.2 μm) and ultra-thin (70–90 nm) cross sections were cut with an OM U2 Ultra Microtome (American Optical Corporation, Reichert Products, Buffalo, NY, USA) using a 45° diamond knife (Diatome, Hatfield, PA, USA). Semi-thin sections for light microscopy were stained with Toluidine Blue as described in Burns (1978) and Mercer (1963) for 30 sec, and photos were taken with a Zeiss Axioplan 2 (Carl Zeiss Microscopy, Jena, Germany) at 40× magnification with 0.63×lens and a digital camera AxioCam HRc (Carl Zeiss Microscopy, Jena, Germany). Images were analyzed with UTHSCSA Image Tool for Windows Version 3.00 (UTHSCSA, Texas, USA 1995–2002). Thickness of the leaf, palisade and spongy mesophyll, and upper and lower epidermis were measured from 10 sections per treatment.

Samples for transmission electron microscopy (TEM) were contrasted with 2% uranyl acetate in 50% ethanol solution for 2 min and in 0.2% lead citrate in 0.1 M sodium hydroxide solution for 2 min (Bozzola and Russell, 1992). Samples were viewed with a Philips Tecnai 10 TEM (FEI, Eindhoven, Netherlands) with an accelerating voltage of 80 kV. 1100–2900x magnification was used to measure mesophyll cells size, and chloroplast and mesophyll surface area exposed to intercellular air space (Sc and Sm). Sc and Sm were calculated according to Evans et al. (1994) by using 2100–4200x magnification for length, thickness and area of chloroplasts, mitochondria and starch grains in the chloroplasts. Finally, 7000–14000x magnification was used for measurements of cell wall thickness and chloroplast distance from the cell wall. All parameters were assessed separately for palisade and spongy mesophyll cells. Based on the measurements we calculated the chloroplast surface area exposed to intercellular air spaces per unit leaf area, mesophyll surface area exposed to intercellular air spaces per unit leaf area, chloroplast area/mesophyll area, and starch grain area relative to chloroplast area.

Photosynthesis Measurements The photosynthetic parameters of the plants during larval feeding were monitored using a set up described in detail earlier (Rasulov et al., 2009; Copolovici and Niinemets, 2010). Light intensity was kept at 200 $nmol\ m^{-2}\ s^{-1}$, temperature at 28°C, and ambient CO_2 concentration was 380–400 $\mu mol\ mol^{-1}$. CO_2 and H_2O concentrations at the chamber in- and outlets were measured with an infra-red dual-channel gas analyzer operated in differential mode (CIRAS II, PP-systems, Amesbury, MA, USA). The rates of net assimilation (A), transpiration (E), and stomatal conductance to water vapour (g_s) were calculated from these measurements according to von Caemmerer and Farquhar (1981).

VOC Sampling and GC-MS Analysis VOC sampling was performed via the outlets of each cuvette every morning with a flow rate of 200 $ml\ min^{-1}$ for 20 min by using a constant flow air sample pump (1003-SKC, SKC Inc.,

Houston, TX, USA). Air was drawn through a multibed stainless steel cartridge (10.5 cm length, 0.3 cm inner diam., Supelco, Bellefonte, PA, USA) filled with Carbotrap C 20/40 mesh (0.2 g), Carbopack C 40/60 mesh (0.1 g), and Carbotrap X 20/40 mesh (0.1 g) adsorbents (Supelco, Bellefonte, PA, USA). In addition, a sample was taken from the air inlet prior to the cuvettes to estimate background VOC concentrations. Adsorbent cartridges were analyzed for lipoxygenase (LOX) pathway products, mono-, homo-, and sesquiterpene emissions with a combined Shimadzu TD20 automated cartridge desorber and Shimadzu 2010 plus GC MS instrument (Shimadzu Corporation, Kyoto, Japan) using a method as detailed in Copolovici et al. (2009) and Toome et al. (2010). The background (blank) VOC concentrations were subtracted from emission samples of the seedlings.

Separate samples were collected from larvae without plants. Emissions of volatile compounds from insects were below the detection limits of our system (range from 1.4 to 5.2 nmol mol^{-1} for all compounds (Copolovici et al., 2009).

Statistical Analysis and Data Handling Experiments were replicated three times with independent samples of plants and larvae. Means were statistically compared by Tukey's test and Student ANOVA *post-hoc* test using ORIGIN 8 (OriginLab corporation, MA, USA) and STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK, USA).

Results

Plant Damage by Herbivory The percentage of total *Alnus* leaf area eaten by the geometrid *C. pusaria* larvae varied between 8 and 17%. Leaf area eaten correlated positively with the number of larvae feeding, which varied from 2 to 8 (Fig. 1a). The amount of leaf dry mass eaten by 2 and 4 larvae was 27.1±2.0 mg DW/larva. For 8 larvae, the amount decreased significantly to 15.0±2.0 mg DW/larva ($P<0.05$ for the comparison between 2 and 4 larvae and 8 larvae), i.e., 8 larvae together were eating about as much as 4 larvae. Leaf mass eaten per larva and larval number correlated negatively (Fig. 1b).

Herbivory Effects on Leaf Structure, Chemistr,y and Photosynthetic Characteristics The average (±SE) leaf dry mass per unit area of *A. glutinosa* leaves was 45±5 $g m^{-2}$, and leaf dry to fresh mass ratio was 0.29±0.02 $g g^{-1}$ (no statistical differences between the insect treatments for either trait, $P>0.1$). Nitrogen content of the leaves varied between 3.6 and 3.8% in control and between 2.3 and 3.2% in the infested plants. Nitrogen content of the bulk leaf material decreased with increase of the amount of leaf area eaten, indicating that larvae were eating primarily the N-rich intercostal leaf areas (Fig. 2).

Chloroplast length and overall cross-sectional area increased by the end of the treatment with increasing degree of *C. pusaria* feeding (Table 1A). As a result, the total chloroplast cross-sectional area relative to mesophyll area increased (Table 1B). In addition, chloroplasts tended to be adhered closer to the cell walls in herbivore-damaged than in the control leaves (Table 1A). In addition, the size of the starch grains and the overall area of chloroplasts covered by starch grains increased with increase of *C. pusaria* feeding (Table 1A).

Net assimilation rate *A* was stable at a level of 16–17 μmol m^{-2} s^{-1} throughout the feeding treatment in both control plants and in those infested with the largest number of larvae (Fig. 3a). Although a small increase of ca 5% to 18–19 μmol m^{-2} s^{-1} was detected after larvae removal (Fig. 3b), this effect was non-significant for the three replicates studied ($P>0.05$) (Fig. 3a). Analogously to the net assimilation rate *A*, stomatal conductance was stable at 65 mmol m^{-2} s^{-1} throughout the study in both control and infested plants (Fig. 3b). No feeding effects on photosynthetic characteristics were detected in the treatments with intermediate herbivory.

VOC Emission Lipoxygenase pathway products (LOX compounds) were the first volatiles induced by *C. pusaria* larval feeding. On the first day of the experiment, plants with 4 larvae emitted significantly more of the lipoxyge-

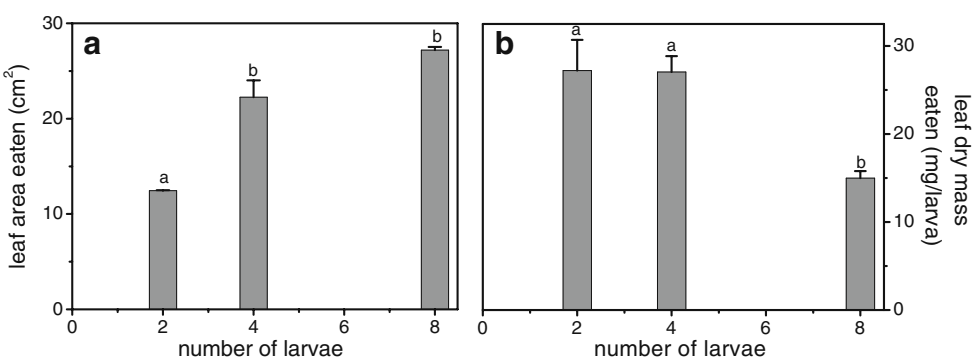


Fig. 1 Leaf area (**a**) and leaf dry mass (**b**) eaten per *Cabera pusaria* larva when different numbers of larvae were exposed to foliage of *Alnus glutinosa*. Measurement: at the end of experiment (d 5). Each data point represents the mean (±SE) of 3 independent replicates. Means were evaluated by Tukey's test, and different letters indicate means that are statistically different at $P<0.05$

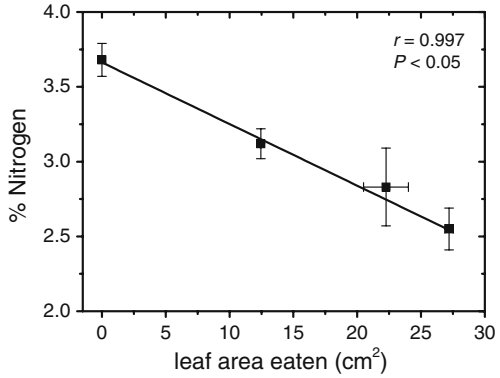


Fig. 2 Leaf nitrogen content (%) in relation to leaf area eaten (cm^2) per *Cabera pusaria* larva when different numbers of larvae were exposed to the foliage of *Alnus glutinosa*. Data show means ± SE of all leaves of 3 independent replicates

nase pathway compounds (*E*)-2-hexenal ($P<0.01$), (*Z*)-3-hexenol ($P<0.01$), 1-hexanol ($P<0.01$), and (*Z*)-3-hexenyl acetate ($P<0.01$) than uninfested plants (Fig. 4). A similar effect was observed in the treatment with 2 larvae. Emissions of the LOX products were maintained at the high level during day two (for comparison of LOX emission rate between day 1 and 4: $P<0.001$, $P<0.05$, $P<0.05$ for 2, 4, and 8 larvae, respectively) (Fig. 5a).

Low emissions of terpenes, close to the baseline level of 0.03 nmol m^{-2} s^{-1}, were observed in control plants. Induction of the monoterpenes α-pinene ($P=0.012$), β-pinene ($P=0.025$), limonene ($P=0.016$), (*E*)-β-ocimene ($P=0.021$), and the sesquiterpene (*E*,*E*)-α-farnesene ($P=0.021$) was observed already on the first day of larval feeding (Figs. 4 and 5). Significant increase of emission of the homoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT) was observed at day 2 of larval feeding ($P>0.3$ for differences between treatments on day 1, $P<0.001$ for day 2, Fig. 6). These emissions had a particularly strong increase on the 3rd day, and a 50% decrease by the 4th day.

On the 1st day after larval infestation, emissions of all induced compounds were not significantly different between the plants treated with different number of larvae ($P=0.95$ for total monoterpenes, and $P=0.78$ for (*E*,*E*)-α-farnesene). Only for LOX emissions, two larvae induced lower emissions than either 4 or 8 larvae ($P=0.008$, Fig. 5). Emissions of monoterpenes decreased on day 2 for the treatment with 2 larvae, but remained constant at the high level for plants with 4 and 8 larvae. By the 4th day, emissions of monoterpenes released by plants with 2, 4, and 8 larvae differed considerably ($P<0.01$, least significant difference of 0.071 nmol m^{-2} s^{-1}).

No significant difference in the release of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene was observed among the treatments with different numbers of larvae during the first 3 days of the experiment ($P>0.1$). However, by the 4th day, emission rates of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene differed significantly among plants with 2, 4, and 8 larvae ($P<0.001$). Feeding by different number of larvae did not cause statistically different emissions of DMNT ($P>0.1$).

Table 1 Effects of *Cabera pusaria* larvae on foliage of *Alnus glutinosa*: anatomical characteristics

Trait	Average ± SE (*N*=10)				*F*	*P*
	Control	2 larvae	4 larvae	8 larvae		
A. Leaf ultrastructure						
cell wall thickness (μm)	0.22 ± 0.02^{a}	0.24 ± 0.02^{ab}	0.32 ± 0.02^{b}	0.23 ± 0.02^{a}	4.2	0.012
chloroplast length (μm)	4.22 ± 0.37^{a}	5.08 ± 0.37^{ab}	5.91 ± 0.37^{bc}	6.69 ± 0.37^{c}	8.3	<0.001
chloroplast thickness (μm)	1.29 ± 0.22^{a}	2.43 ± 0.22^{b}	2.1 ± 0.22^{ab}	2.73 ± 0.22^{b}	8.0	<0.001
chloroplast distance from cell wall (μm)	0.35 ± 0.05^{b}	0.17 ± 0.05^{ab}	0.24 ± 0.05^{ab}	0.14 ± 0.05^{a}	3.6	0.023
chloroplast area (μm^2)	4.24 ± 1.33^{a}	9.66 ± 1.33^{b}	9.79 ± 1.33^{b}	13.93 ± 1.33^{b}	8.9	<0.001
mitochondrion length (μm)	1.27 ± 0.12^{a}	0.91 ± 0.15^{a}	1.05 ± 0.12^{a}	1.05 ± 0.16^{a}	1.5	0.23
mitochondrion thickness (μm)	1.08 ± 0.10^{b}	0.67 ± 0.12^{ab}	0.64 ± 0.10^{a}	0.92 ± 0.13^{ab}	4.2	0.012
ratio of starch grain area to chloroplast area (%)	3.15 ± 2.45^{ab}	0.06 ± 2.74^{a}	11.52 ± 2.45^{b}	9.60 ± 2.45^{ab}	4.4	0.010
Trait	Average ± SE (*N*=6)				*F*	*P*
	Control	2 larvae	4 larvae	8 larvae		
B. Whole leaf integrated traits						
chloroplast surface area exposed to intercellular air space (Sc)	2.82 ± 0.47^{a}	3.16 ± 0.51^{a}	4.66 ± 0.47^{a}	4.56 ± 0.51^{a}	3.8	0.024
mesophyll surface area exposed to intercellular airspace (Sm)	4.47 ± 0.66^{a}	5.40 ± 0.71^{a}	6.68 ± 0.66^{a}	6.54 ± 0.71^{a}	2	0.095
Sc/Sm	0.63 ± 0.04^{a}	0.6 ± 0.04^{a}	0.69 ± 0.04^{a}	0.69 ± 0.04^{a}	1.45	0.254
starch grain area/chloroplast area	0.015 ± 0.012^{ab}	0.00073 ± 0.013^{a}	0.130 ± 0.013^{c}	0.0529 ± 0.013^{b}	19.8	<0.001

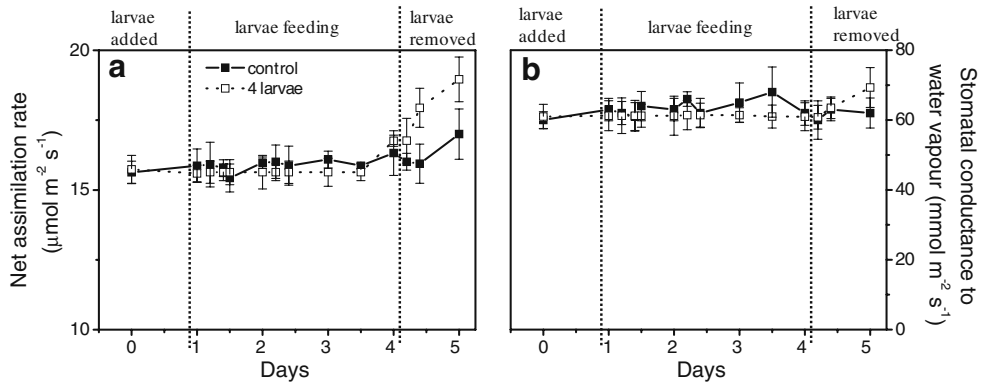


Fig. 3 Net assimilation rate (**a**) and stomatal conductance to water vapor (**b**) in seedlings of *Alnus glutinosa* without insects—control (*filled square*) and with four larvae of *Cabera pusaria* (*white square*). Measurements were conducted at a quantum flux density of 200 μmol m^{-2} s^{-1}, air temperature of 28°C, and at ambient CO_2 concentrations of 380±20 μmol mol^{-1}. Data are expressed per unit projected leaf area. Each data point is the mean (±SE) of 4 independent replicate experiments. At d -1, plants without insects were measured, at d 0 insects were added, and after d 4 the insects were removed

We observed that the kinetics of (*E*,*E*)-α-farnesene emissions tended to be biphasic (Fig. 6). (*E*,*E*)-α-Farnesene emissions of plants with 8 larvae were not statistically different (P=0.4) between day 1 and 2, but between day 3 and 4 emissions differed considerably (P<0.1). These biphasic kinetics were reproduced in all replicates and with different numbers of larvae. In addition, a biphasic model (cubic) fitted the data (r^2=0.84) better than a monophasic (parabola) model (r^2=0.59). On the 4th day, larvae were removed from plants, and by the 5th day, the emission of all compounds decreased to the level close to the start of the experiment (Fig. 6).

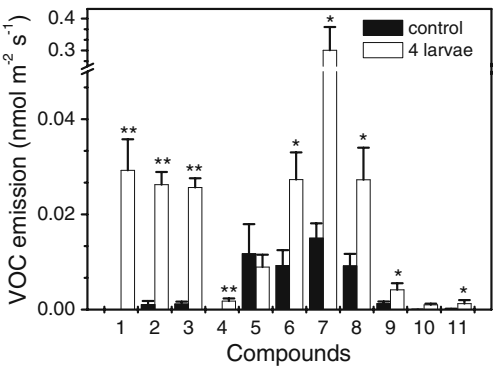


Fig. 4 Volatile organic compound (VOC) emissions from herbivore-attacked (*white bars*) and control (*black bars*) *Alnus glutinosa* plants (means + SE) on the first day of insect feeding (treatment with 4 larvae). Asterisks above the compound numbers indicate significant differences in VOC emissions between control and larvae-infested sample for each compound (*: P>0.05; **: P> 0.01). In other cases, VOC emissions were not significantly different among the treatments (ANOVA *post-hoc* test). Compounds: (*1*) 1-hexanol, (*2*) (*E*)-2-hexenal, (*3*) (*Z*)-3-hexenol, (*4*) (*Z*)-3-hexenyl acetate, (*5*) 6-methyl-5-hepten-2-one, (*6*) limonene (*7*) α-pinene, (*8*) β-pinene, (*9*) (*E*)-β-ocimene, (*10*) DMNT, (*11*) (*E,E*)-α-farnesene

Quantitative Relationships between VOC Emission and Damage by Herbivory Emission of total monoterpenes, LOX products, and (*E*,*E*)-α-farnesene on day 4 were positively correlated with the leaf area eaten by larvae (Fig. 7). The trends were exponential with good correlation coefficients (r>0.98). However, the emission rates of DMNT did not increase with an increase of the number of larvae (r=0.57, P>0.1).

Discussion

Herbivory Effects on Leaf Structure, Chemistry, and Photosynthetic Characteristics Foliage nitrogen content decreased with increase of damage (Fig. 2). As nitrogen content of the major veins is less than that of the intercostal areas (Niinemets et al., 2007), reduction of whole leaf nitrogen content may reflect the selective feeding of intercostal N-rich mesophyll. It is also known that lower leaf quality reduces the rate of larval growth and development (Tikkanen and Julkunen-Tiitto, 2003; Silla et al., 2008). Thus, lower leaf nitrogen content has been suggested to be a direct defense strategy reducing insect herbivory (Cornelissen and Fernandes, 2001).

Previous studies have shown that plant photosynthetic responses to biotic stresses such as herbivory or rust can range from impairment, no net effect, to a stimulating effect (Paige, 1999; Delaney, 2008; Delaney et al., 2008). In our study, there was no net effect on photosynthetic rate per unit leaf area (Fig. 3). This is surprising, given the overall reduction of leaf nitrogen content as the result of selective feeding of photosynthetically active N-rich tissues. Given this reduction, a constant net assimilation rate actually reveals compensatory responses in herbivore-damaged leaves relative to foliage of

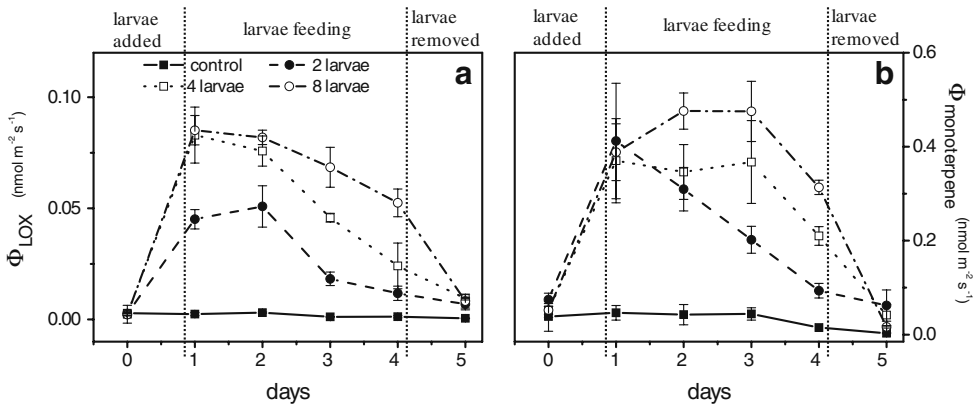


Fig. 5 Emission time-courses (in nmol m^{-2} s^{-1}) of total LOX products (**a**) and monoterpenes (**b**) in the case of healthy leaves and in leaves fed by different numbers of larvae of *Caberia pusaria*. Treatments and data presentation as in Fig. 3

the control plants. In our study, this compensatory response was associated with the overall increase in chloroplast size (Table 1A). Additionally, adherence of chloroplasts closer to the cell wall surface can reduce the CO_2 diffusion resistance from substomatal cavities to chloroplasts (Evans et al., 2009; Niinemets et al., 2009), thereby further compensating for the reduction of N-rich mesophyll.

It has been suggested that compensatory photosynthesis usually is found when herbivory alters the strength of the source-sink relationships, thus increasing the relative or absolute demand of sinks for carbohydrates (Delaney et al., 2008). In our study, we actually observed accumulation of starch in the herbivore-damaged plants (Table 1), suggesting that sink demands were reduced. Reduction of plant growth activity is common in feeding-damaged plants (Poveda et al., 2003) and can reflect the shift of metabolism towards synthesis of protective high molecular mass secondary compounds such as tannins (Bernays et al., 1989; Dudt and Shure, 1994; Hattenschwiler and Schafellner, 1999) in agreement with the largely discredited carbon/nutrient balance hypothesis (Reichardt et al., 1991). Although no changes in net assimilation rates were observed, these data collectively suggest that herbivory-driven modifications in leaf ultrastructure and in sink-source balance occurred during the experiment.

Elicitation of LOX Products When *C. pusaria* larvae damaged leaves of *A. glutinosa*, the production of lipoxygenase (LOX) pathway compounds was the first signal of the induction of a plant stress response, already achieving the maximum emission rate on the first day of feeding. This result is consistent with the observations that oxidative stress and electrolyte leakage are induced by insect damage (Bruinsma et al., 2010). Emissions were present throughout the feeding treatment, reflecting continuous damage of foliage during feeding, while emissions decreased drastically after larvae were removed and the stress was relieved (Figs. 4 and 5a).

Previously, LOX products emission has been shown to scale with the severity of water (Capitani et al., 2009) and ozone (Beauchamp et al., 2005) stresses. Here, we found strong correlation of maximum LOX emission rates with the number of feeding larvae and with the amount of leaf area eaten (Fig. 7b), providing evidence that quantitative stress dose (number of larvae feeding) vs. stress response relationships can also be determined for biotic stresses.

Induction of Ubiquitous Terpenoid Emissions Leaves of *A. glutinosa* do not accumulate terpenes (monoterpenes, sesquiterpenes, or terpenoid derivatives), and do not emit any of these compounds under non-stressed conditions (Isebrands et al., 1999; Lindfors et al., 2000). In control plants analyzed in our study, terpenoid emissions were very low, close to the detection limit, further confirming that *A. glutinosa* is no constitutive terpene emitter.

The feeding by *C. pusaria* caterpillars resulted in induced emissions of ubiquitous monoterpenoids on the first day (Fig. 5b). The emissions peaked at days 2 and remained high in the following days of feeding. Analogously, increases in ubiquitous terpene emissions within the first 2 h of feeding and greater emission following the night period have been observed in a field experiment with hybrid aspen during feeding by larvae of the winter autumn moth *Epirrita autumnata* (Schaub et al., 2010). Continuous increase of ubiquitous emissions was observed until day 3 for experiments with more than 4 larvae per plant (Fig. 4b). In a similar manner, in herbivory experiments with *Chrysomela populi* leaf beetles, the rate of monoterpene emission from poplar leaves increased for the first 4 days of feeding (Brilli et al., 2009). However, emission rates already were strongly reduced by day 1 in our study in the treatment with fewer larvae (Fig. 5b). Overall, strong positive correlations were found between the rate of ubiquitous monoterpene emissions and the number of feeding larvae and the amount of leaf area eaten, again demonstrating a quantitative stress dose vs. plant response.

Induction of Stress-Specific Terpenes While emissions of LOX and ubiquitous monoterpenes were detected 1 day after larval feeding started, emissions of the monoterpene

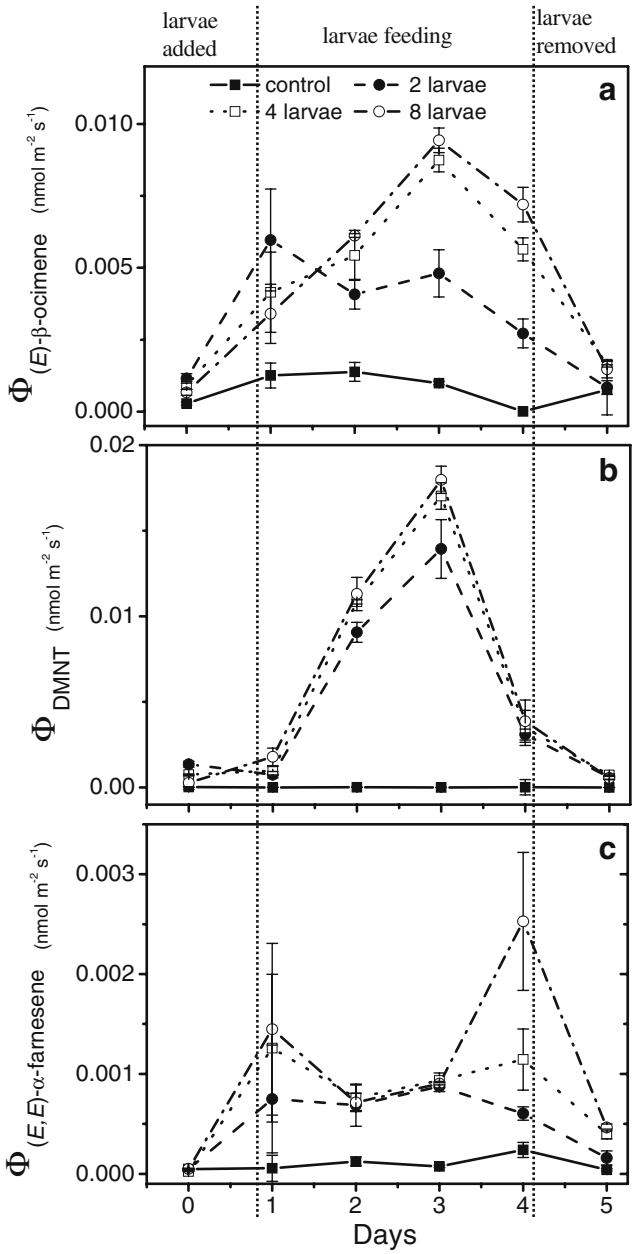


Fig. 6 Temporal variation of (*E*)-β-ocimene (**a**), DMNT (**b**), and (*E*,*E*)-α-farnesene (**c**) emissions (in nmol m^{-2} s^{-1}) from *Alnus glutinosa* plants fed by *Caberia pusaria* larvae. Treatments and data presentation as in Fig. 3

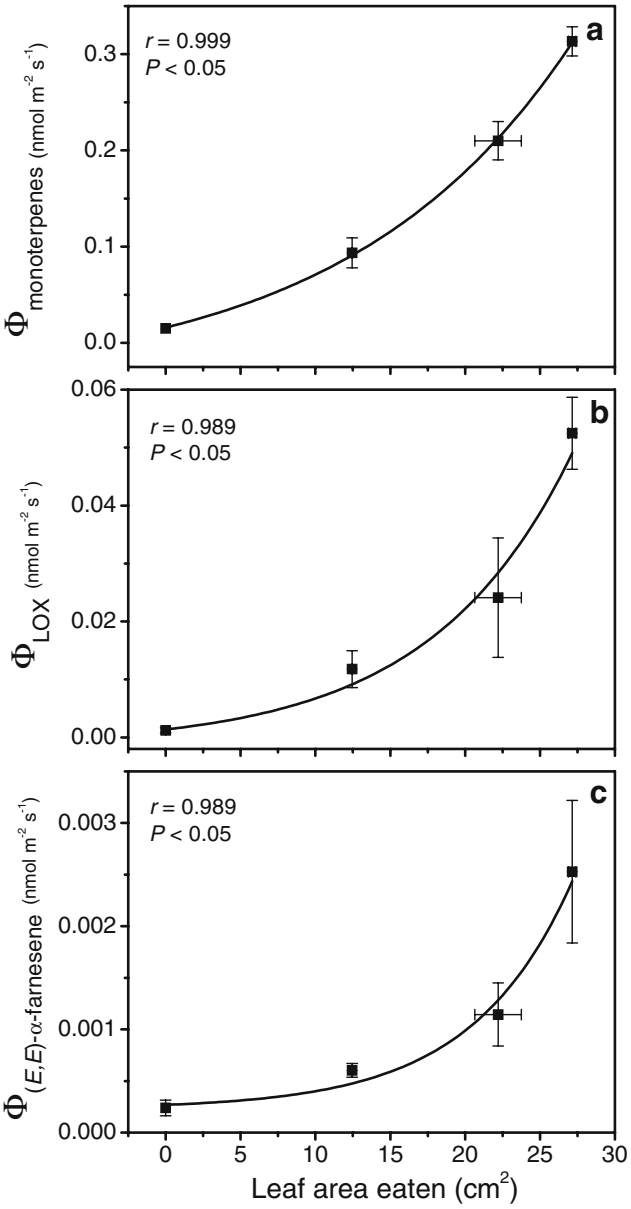


Fig. 7 Correlations of total monoterpenes (**a**), LOX products (**b**), and (*E*,*E*)-α-farnesene (**c**) emissions (in nmol m^{-2} s^{-1}) with the amount of leaf area of *Alnus glutinosa* eaten by *Caberia pusaria* larvae in the 4th day of feeding. Data were fitted by non-linear regressions in the form $y = a \cdot e^{b \cdot x}$. All other conditions and data presentation as in Fig. 3

(*E*)-β-ocimene, the homoterpene DMNT, and the sesquiterpene (*E*,*E*)-α-farnesene were observed only on the 2nd day. Hence, biosynthesis of these compounds apparently starts later than those of LOX products and other ubiquitous monoterpenes (Brilli et al., 2009).

We observed that the kinetics of (*E*,*E*)-α-farnesene emissions tended to be biphasic (Fig. 6). In previous studies, multiphasic emission responses of LOX volatiles and methanol after stress have been demonstrated (Beauchamp et al., 2005). Statistical comparisons among days did not confirm the biphasic response, but nevertheless the kinetics were reproducible, and fitting all data by biphasic function yielded a higher degree of explained variance. Interestingly, the first peak of (*E*,*E*)-α-farnesene emissions co-occurred with the onset of enhanced emissions of ubiquitous monoterpenes and (*E*)-β-ocimene, while the second peak followed the peak in DMNT emissions (Fig. 6). There is evidence that different terpenoid classes are synthesized in different subcellular compartments, with the production of sesquiterpenes being traditionally thought to be located in the cytosol, while the

production of monoterpenoids is confined to the plastids (Nagegowda, 2010). Furthermore, enzymes of the mevalonate (MVA) pathway are present in the cytosol, while typically the genes coding for enzymes of the mevalonate pathway are presented in the chloroplast and in the mitochondrial compartments (Lee and Chappell, 2008). This suggests different pathways for synthesis of (*E*,*E*)-α-farnesene and (*E*)-β-ocimene. Nevertheless, there are numerous examples of coordination of expression of nuclear and chloroplastic genes during biotic stress (e.g., De Coninck et al., 2010). Furthermore, there is now conclusive evidence of plastidial synthesis of sesquiterpenes as well (Sallaud et al., 2009). Thus, it is tempting to speculate that the first peak of (*E*,*E*)-α-farnesene emission coinciding with (*E*)-β-ocimene emission reflects activation of chloroplastic sesquiterpene synthesis, while the second peak coinciding with DMNT reflects activation of the cytosolic pathway.

Previous studies demonstrate that (*E*)-β-ocimene synthase is induced by insect herbivory in *Medicago truncatula* leaves (Navia-Gine et al., 2009), and that induction of expression of terpene and sesquiterpene synthases genes are involved in the overall defense response in *Oryza sativa* plants (Yuan et al., 2008). Elicitation of defense isoprenoid emissions can further elicit defense genes in undamaged lima bean leaves (Arimura et al., 2000), and biosynthesis of different β-ocimene isomers are almost exclusively fuelled by the recently assimilated photosynthetic carbon according to the plastidial 2-C-methyl-D-erythritol 4-P (MEP) pathway (Arimura et al., 2008). On the other hand, in bean plants, the emission of induced (*E*)-β-ocimene was reduced when the whiteflies *Bemisia tabaci* attacked spider mite-infested plants (Zhang et al., 2009). Although our data cannot be used to resolve the cause/effect relationships in the sequence of various terpenoids emitted, we suggest that the secondary peak in (*E*,*E*)-α-farnesene emissions is associated with systemic induction of DMNT in undamaged leaves of an herbivore-infested plant.

After larvae were removed, emission of (*E*,*E*)-α-farnesene and (*E*)-β-ocimene rapidly decreased until reaching the level of non-stressed plants (Fig. 6). Previous studies have demonstrated that specific elicitors in the regurgitant of the caterpillar enhance the amount of several released volatiles (Halitschke et al., 2000; Rose and Tumlinson, 2005), Thus, reductions in volatile emissions after feeding and removal of larvae can reflect the lack of these elicitors. However, the emissions of DMNT started to decrease already during insect feeding, suggesting a different mechanism of induction. Different mechanisms of action are consistent with previous experiments employing various elicitors. Bartram et al. (2006) demonstrated that insect feeding resulted in induction of volatile release predominantly via activation of the MVA pathway, while exposure to fungal elicitor alamethicin stimulated the production of DMNT mainly via the MEP pathway.

As with ubiquitous terpenes, the emission rates of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene were quantitatively related to the strength of biotic stress (Fig. 7). However, no such pattern was visible in the case of DMNT. We suggest that the quantitaties of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene can be important for attraction of natural enemies of the herbivores, as the attraction effect of certain compounds may depend on their relative amounts compared to other volatiles (Beyaert et al., 2010).

In summary, these data provide evidence that biotic stress effects on plant VOC emissions are quantitative for ubiquitous monoterpenes, (*E*)-β-ocimene, and (*E*,*E*)-α-farnesene. To our knowledge, this quantitative relationship between plant VOC emission and extent of plant damage by herbivory has not been shown in deciduous trees. In contrast to the other compounds, DMNT emissions were not quantitatively related to the extent of herbivory stress. Thus, we hypothesize that DMNT can serve as a qualitative signal for predators and parasitoids that conveys the information "presence of herbivores", while the emission of mono- and sesquiterpenes or their amounts relative to other volatiles provides information about the abundance of the herbivores. Parasitoid and predator attraction experiments are needed to test this hypothesis.

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Differential Response in Foliar Chemistry of Three Ash Species to Emerald Ash Borer Adult Feeding

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Abstract The emerald ash borer (EAB; *Agrilus planipennis* Fairmaire; Coleoptera: Buprestidae), is an exotic wood-boring beetle that has been threatening North American ash (*Fraxinus* spp.) resources since its discovery in Michigan and Ontario in 2002. In this study, we investigated the phytochemical responses of the three most common North American ash species (black, green, and white ash) in northeastern USA to EAB adult feeding. Black ash was the least responsive to EAB adult feeding in terms of the induction of volatile compounds, and levels of only two (indole and benzyl cyanide) of the 11 compounds studied increased. In green ash, levels of two [(*E*)-*β*-ocimene and indole] of the 11 volatile compounds studied were elevated, while the levels of two green leaf volatiles [hexanal and (*E*)-2-hexenal] decreased. White ash showed the greatest response with an increase in levels of seven of the 11 compounds studied. Qualitative differences among ash species were detected. Among the phenolic compounds detected, ligustroside was the only one detected in all three species. Oleuropein aglycone and 2 unidentified compounds were found only in black ash; coumaroylquinic acid and feruloylquinic acid were detected only in green ash; and verbascoside hexoside was detected only in white ash. EAB adult feeding did not elicit or decrease concentrations of any selected individual phenolic compounds. However, although levels of total phenolics from black and green ash foliage were not affected by EAB adult feeding, they decreased significantly in white ash. EAB adult feeding elevated chymotrypsin inhibitors in black ash. The possible ecological implications of these findings are discussed.

Key Words *Agrilus planipennis* · *Fraxinus* · Plant defense · Phenolics · Volatile organic compounds · VOCs · Coleoptera · Buprestidae

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Introduction

Many volatile and non-volatile phytochemicals have various ecological and physiological functions in interactions with biotic and abiotic factors. Some adversely affect colonization or development by herbivores (Berenbaum 1995; Duffey and Stout 1996), while some function as herbivore attractants or attractants for natural enemies of herbivores (Dicke et al. 1990; Turlings et al. 1991). In response to herbivore damage, many of these compounds can accumulate in plant tissues (Karban and Baldwin 1997; Chen et al. 2008, 2009) with significant effects on herbivore interactions with conspecifics and heterospecifics (Agrawal and Karban 2000). For instance, increased density of pigment glands (containing terpenoids such as gossypol) of cotton leaves induced by strawberry mites, *Tetranychus turkestani* Ugarov & Nikolskii (Prostigmata: Tetranychidae), significantly reduced beet armyworm growth [*Spodoptera exigua* Hübner (Lepidoptera: Noctuidae)] (Agrawal and Karban 2000). Beet armyworm larvae also can distinguish between previously damaged and intact

(control) cotton leaves and will consume more control leaves than previously damaged leaves in dual-choice tests (McAuslane et al. 1997).

The exotic emerald ash borer (EAB; *Agrilus planipennis* Fairmaire; Coleoptera: Buprestidae) has been threatening North American ash (*Fraxinus* spp.) trees since its discovery in Michigan and Ontario in 2002 (MSU 2010). Although North American ash species, such as black ash (*F. nigra* Marshall), green ash (*F. pennsylvanica* Marshall), and white ash (*F. americana* L.) are all susceptible to EAB compared to the Asian ash (e.g., Manchurian ash, *F. mandshurica* Ruprecht) (Rebek et al. 2008), evidence points to inter-specific variation in constitutive foliar chemistry among these North American species (Pureswaran and Poland 2009). Differences in feeding and infestation preference in laboratory studies appear to be associated with such phytochemical variability (Pureswaran and Poland 2009, Chen and Poland 2010). Intact black, green, and white ash release many volatiles that trigger electro-antennal responses of EAB adults (Rodriguez-Saona et al. 2006; de Groot et al. 2008) and possess a wide variety of phenolics (Eyles et al. 2007) and protease inhibitors (Chen and Poland 2009b) that may confer resistance against EAB. Phenolics generally denature plant proteins and thus reduce nutritive values of plants (Dudt and Shure 1994; Ossipov et al. 2001). Phenolics also can inhibit the absorption of key amino acids. Many protease inhibitors are involved in herbivore defense (Ryan 1990; Zavala et al. 2004). Protease inhibitors impair herbivore growth by eventual depletion of essential amino acids that result from an herbivore's compensatory production of digestive proteases (Broadway and Duffey 1986).

Many phytochemicals can be induced by herbivore damage. However, little is known about the physiological response of North American ash species to EAB adult feeding. Differential responses of ash species to EAB adult feeding may change the dynamics of ash foliar phytochemicals, which can further affect the behavior of EAB, other herbivores of ash trees, and potential natural enemies of EAB larvae. Elucidating inter-specific variation among these compounds also might contribute to understanding differences in feeding and infestation preference among North American species. Our objectives in this study were to determine: (1) the quantity and composition of volatile organic compounds (VOCs), polyphenols, and trypsin and trypsin inhibitors of the three ash species in response to EAB adult feeding; (2) if the magnitude of induction differs among the three species.

Methods and Materials

Ash Seedlings Green, white, and black ash seedlings (2-yr-old and ca. 30–50 cm tall) were purchased from Lawyer Nursery Inc. (West Plains, MT, USA) in February, 2009. Trees were stored in a dark room at 4°C until potting. Seedlings were potted in TPOT2 tree pots (width: 15 cm; height: 41 cm; volume: 6.23 L; Stuewe & Sons, Inc., Corvallis, OR, USA) using Fafard #52 soil (BFG Supply Co., Burton, OH, USA) as planting medium. They were grown during April-May, 2009 (ca. L14: D10), in a greenhouse set at approximately 25±2°C. The greenhouse is located at the Tree Research Center, Michigan State University, East Lansing, MI, USA. Seedlings were fertilized once every 3-4 day with approximately 2 L nutrient solution containing 200 ppm N, 60 ppm P, 150 ppm K, 80 ppm Ca, 40 ppm Mg, 60 ppm S, 0.15 ppm Cu, 4 ppm Fe, 0.8 ppm Mn, and 0.32 ppm Zn.

EAB Adult Females EAB adult females were collected from 90-cm long logs cut from naturally infested ash trees felled at the end of 2008 and early 2009 in Ingham County, MI, USA. Logs were held in cold storage at 4°C until needed, then removed to allow beetles to emerge. Beetles were separated by sex and kept in 295 ml plastic beverage containers with an evergreen ash, *F. uhdei* (Wenzig) Linglesh, leaf in a vial of water for feeding. Containers with beetles were stored in growth chambers (25°C) with a photoperiod of 14:10 hL:D, and >70% relative humidity. Leaves were replaced twice a week, until needed for experiments.

Headspace Leaf Volatile Collection and Analysis Constitutive headspace volatile profiles of the three species used were investigated in Pureswaran and Poland (2009). Headspace leaf volatiles of EAB damaged leaves (a whole branch from "induced treatment", see below) and those without EAB damage (a whole branch from "control treatment") of the same ash species attached to live seedlings were collected and compared in this study.

EAB treated leaves were prepared by caging a branch bearing 5–8 mature compound leaves within a nylon sleeve with both ends tied. Mature leaves were chosen because EAB adults are known to prefer mature over young foliage (Chen and Poland 2009b). To simulate common levels of foliage damage in nature by EAB, adults (personal observations), ten 8- to 10-d-old adult females were placed inside the sleeve and allowed to feed for 48 h. Adult females then were removed and frass gently brushed from the leaves. Approximately 5–10% of the foliage was visually estimated to be consumed within the 48 h ("induced treatment"). Control leaves also were enclosed by a sleeve but without beetles ("control treatment").

The volatile compounds of the whole branch, either from "induced treatment" or "control treatment", subsequently

were collected with a push-pull system described in Rodriguez-Saona et al. (2006). Briefly, the branch was enclosed in a 4 L glass cylinder (height: 30.5 cm; diam: 15.2 cm; Analytical Research Systems, Inc., Gainesville, FL, USA). Activated charcoal-purified air generated by a pump entered the top of the glass cylinder at a rate of 2 L/min. Half of the air with a rate of 1 L/min passed through a volatile collection trap (VCT) located 5 cm above the base of the cylinder by a vacuum pump. The VCT was filled with 30 mg Alltech Super-Q absorbent material. Volatiles were collected in the laboratory under 25±1°C with about 40% RH for 24 h. The room was illuminated with four 40 W fluorescent light tubes. At the end of volatile collection, the aerated portion of the seedling was clipped, and dry weights (oven-dried at ca. 60 C) of aerated tissue were recorded. Each treatment (EAB vs. control) was replicated 8 times. Volatile collection was conducted separately by species (i.e., black, green, and white ash) due to the limitation of available volatile collection systems. Volatile collection was conducted approximately 4 week after potting of the seedlings.

Methods of volatile extraction and analysis are described in Chen and Poland (2009a). Collected volatiles were extracted from the Super-Q adsorbent by flushing with 250 μl of pentane-hexane (both HPLC grade; purity: pentane >99%; hexane: ≥ 95%) mixture (1.5:1 ratio) containing 1 ng μl^{-1} of heptyl acetate (internal standard; purity >98%) with a stream of nitrogen gas. Two μl of each sample were injected into a Thermo Scientific Trace GC gas chromatograph equipped with a DSQ-II MS mass spectrometer and a 30 m × 0.25 mm id, 0.25 μm thick TR-1MS column by using splitless injections and helium as the carrier gas. The oven temperature began at 60°C for 1 min, increased by 10°C/min to 190°C, then by 35°C/min to 300°C, and held for 5 min. The following 11 compounds were detected consistently: hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, (*Z,E*)-α-farnesene, α-humulene, β-caryophyllene, cubenene, (*E*)-β-ocimene, indole, benzyl cyanide, and nonanal. They were quantified based on comparison of peak areas with that of the internal standard and calibration curves generated with synthetic standards. The purity of these synthetic standards was 98% (hexanal), 98% [(*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, (*Z,E*)-α-farnesene, α-humulene, β-caryophyllene, and cubenene], 70% [(*E*)-β-ocimene and indole], 99% (benzyl cyanide), and 95% (nonanal). The amount of each compound of interest was calculated in ng g^{-1} dry weight of tissue. All reagents and solvents used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Determination of Non-volatile Defensive Compound We examined the following non-volatile foliar defense responses to EAB adult feeding: soluble phenolics (total and individual), trypsin, and chymotrypsin inhibitors. Analyses were run for both treatments, EAB damaged (induced) and no damage (control), for all species examined above. The preparation of EAB feeding was as described in the preceding experiment. Mature leaves with (a branch from "induced treatment") and without (a branch from "control treatment") EAB feeding damage were cut and flash frozen in liquid nitrogen approximately 4 week after seedling potting. Samples then were ground to a fine powder with a mortar and pestle in liquid nitrogen.

Extraction of Soluble Phenolics Phenolics were extracted according to Eyles et al. 2007. Briefly, ~80–120 mg of homogenized foliage tissue were extracted twice overnight in the dark at 4 C with 500 μl of 100% MeOH. Extracts were pooled and transferred into a 1.5-ml microcentrifuge tube and centrifuged (12,000xg for 5 min) to remove solids. Samples were stored at -20 C and used in subsequent HPLC analyses.

Analysis and Identification of Soluble Phenolics HPLC-ESI-MS (Varian 500 MS; Palo Alto, CA, USA) in parallel with a PDA detector (Varian ProStar 335) were employed to detect and identify phenolics. Chromatographic separation of phenolics was carried out using a Waters Xterra™ RP18 analytical column (3.9 μm). The binary mobile phase consisted of water/acetic acid (99.9:0.1 v/v) (eluant A) and methanol/acetic acid (99.9:0.1 v/v) (eluant B) with a flow rate of 1 ml/min. The gradient was as follows (percentages refer to proportions of eluant B): 5–15% (0–15 min); 15–30% (15–35 min); 30–40% (35–40 min); 40–60% (40–50 min); 60–90% (50–55 min); 90–100% (55–60 min). The injection volume for all samples was 10 μl. Compound separation was achieved by post-column splitting (1:1), and passing the LC effluent through the PDA Detector (scanning range, 200–400 nm) and an electrospray source (parameters below). The ESI was in negative ion mode. The MS detector was optimized to obtain maximum yields of $[M-H]^-$ ions of apigenin, luteolin, rutin, oleuropein, tyrosol, and verbascoside standards. The optimized MS parameters were: capillary voltage, -80 V; needle voltage, -5 kV. The atmospheric pressure ionization (API) parameters were as follows: API nebulizing gas (air) 25 psi; API drying gas (nitrogen) 15 psi at 350 C. Survey scan was set to detect molecules between 50–1,000 m/z. Each treatment was run using both the TurboDDS scan type in the enhanced scan mode and full scan mode. The TurboDDS trigger threshold was set to 20,000 counts (parent ion counts). In the TurboDDS scan mode, MS scan parameters were set on-the-fly by the instrument to detect the most abundant parent ions. If TurboDDS was triggered, the mode temporarily switched

to MSn mode to perform daughter scans of the putative parent ions. The TurboDDS trigger threshold for daughter ions was 20,000, 2,000, and 200 ions for MS2, MS3, and MS4, respectively. Full scan parameters were set following the conditions mentioned above. Full scan chromatograms were overlaid with the PDA chromatogram traces at 280 nm to match $[M-H]^-$ parent ions to λ_{max} of individual phenolics. PDA data of individual compounds were analyzed using PolyView software (Varian). Phenolics were identified based on the congruence of parent and daughter ions, λ_{max}, and order of time compared to the published literature (Eyles et al. 2007). Data acquisition and processing were performed using MS Workstation 6 (Varian).

Quantitation and Analysis of Soluble Phenolics with HPLC-UV Quantitation of identified phenolics was performed using HPLC-UV. HPLC-UV analyses were performed on an Alliance 2690 separation module (Waters, Milford, MA, USA) equipped with an autosampler and a 996 PDA (Waters). The autosampler and column temperatures were set to 4 and 30 C, respectively. The binary mobile phase consisted of water/acetic acid (98:2, v/v) (eluant A) and methanol/acetic acid (98:2, v/v) (eluant B), with a flow rate of 1 ml/min. The elution program matched the conditions used for HPLC-ESI-MS-PDA analysis mentioned above. The injection volume for all samples was 10 µl. Samples were scanned in the range 200–400 nm. Individual compounds were quantified in mg g^{-1} FW. The identity of hydroxytyrosol, verbascoside, oleuropein, rutin, apigenin, and luteolin were unequivocally confirmed by spiking samples with the respective standards and matching retention times and UV maxima. Due to a lack of external standards for all compounds, samples were quantified following the method of Eyles et al. 2007. Compounds were expressed as hydroxytyrosol equivalents for hydroxytyrosol hexoside and tyrosol hexoside, verbascoside equivalents for verbascoside hexoside, oleuropein equivalents for elenolic acid derivative, ligustroside, nuzhenide, and oleuropein aglycone, apigenin equivalents for apigenin rutinoside, and luteolin equivalents for kaempherol rutinoside and kaempherol rhamnosylhexoside. Quantitation of individual compounds was carried out by comparing peak areas at 280 nm against a standard curve of the equivalent external standard. Total peak areas were taken to represent total phenolics (Bonello and Blodgett 2003; Blodgett et al. 2005). Total and individual peak areas at 280 nm for all species and treatment combinations were normalized to 100 mg dry weight.

Trypsin and Chymotrypsin Inhibitor Extraction and Analysis Trypsin and chymotrypsin inhibitors were extracted with a 10 mM Tris buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), a solution modified from Chen et al. (2007). The mixture was centrifuged at 10,000 g for 10 min. The supernatant was used for assay. The trypsin and chymotrypsin inhibitor assays followed Walsh and Wilcox (1970), and Broadway (1993) using *N*α-*p*-tosyl-L-arginine methyl ester (TAME) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as substrates for trypsin and chymotrypsin determination, respectively. Each treatment was replicated 8 times.

Statistical Analysis Concentrations of volatile and non-volatile foliar compounds were compared individually between treatments using independent *t*-tests. Concentrations of volatiles and phenolics were square root transformed to homogenize variance, while no transformation was necessary for protease inhibitor data. All analyses were conducted in SAS with α=0.05 (SAS Institute 1999).

Results

Foliar Volatile Compound Responses to EAB Adult Feeding Induction of foliage volatile compounds by EAB adult feeding in black, green, and white ash is shown in Table 1. The most abundant volatiles were the three green leaf volatiles, hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate, and a sesquiterpene, *Z,E*-α-farnesene, irrespective of species. Release of indole and benzyl cyanide by black ash was significantly induced, over 7-fold. Emanation of indole and (*E*)-β-ocimene (monoterpene) of green ash was elevated, while two green ash leaf volatiles, hexanal and (*E*)-2-hexenal, decreased. Release of seven volatiles of white ash, (*Z,E*)-α-farnesene, α-humulene, β-caryophyllene, cubenene, (*E*)-β-ocimene, indole, benzyl cyanide, increased. The magnitude of increase ranged from 5-fold [(*Z,E*)-α-farnesene] to over 100-fold (benzyl cyanide). No other compound of the three species was significantly affected by EAB adult feeding.

Foliar Non-volatile Compound Responses to EAB Adult Feeding HPLC-UV chromatographic profiles of control (undamaged) black, green, and white ash are shown in Fig. 1. Tentatively identified and characterized phenolics are presented in Table 2. Only qualitative differences among species were detected (Tables 2, 3). A total of six, 13, and 10 phenolics were detected in black, green, and white ash, respectively. Among those, ligustroside was the only one detected in all three species. Oleuropein aglycone and 2 unidentified compounds (peak # 2 and 4) were found

Table 1 Volatile compound concentrations (mean±SE ng g^{-1} dry foliage tissue) of three ash species with undamaged leaves (constitutive) and leaves induced by emerald ash borer (EAB) adult feeding. N=8 in all cases

Compound	Black ash		Green ash		White ash	
	Constitutive	Induced[a]	Constitutive	Induced[a]	Constitutive	Induced[a]
1. Hexanal	609.3±63.1	464.5±82.2	1,118.6±140.0	718.3±126.2*	3,703.5±955.9	2,304.8±416.9
2. (E)-2-hexenal	160.0±14.3	134.9±22.0	281.9±33.7	185.4±29.0*	927.6±243.6	579.7±98.2
3. (Z)-3-hexenyl acetate	502.1±214.1	214.8±49.9	54.2±13.9	42.9±10.3	118.0±40.0	474.2±204.7
4. Z,E-α-farnesene	498.6±76.8	506.6±53.8	49.3±13.6	71.0±8.9	25.6±11.3	134.0±47.4**
5. α-humulene	5.64±1.3	6.5±2.6	0.8±0.2	2.1±0.7	4.6±1.0	33.3±13.2*
6. β-caryophyllene	31.4±6.4	35.2±12.2	2.4±0.7	7.0±2.4	19.0±5.30	167.5±61.0**
7. cubenene	47.2±13.0	78.5±33.1	14.5±4.8	22.3±11.8	10.5±2.4	79.0±33.0*
8. (E)-β-ocimene	77.1±17.1	133.3±41.1	7.6±1.1	16.0±2.2**	14.3±3.5	121.9±49.2*
9. Indole	3.6±0.5	25.3±8.9*	2.6±1.2	9.3±2.3**	2.1±1.0	21.2±8.1*
10. Benzyl cyanide	38.6±10.3	254.6±60.4**	1.7±0.7	3.9±1.1	0.5±0.2	63.6±30.3**
11. Nonanal	1.2±0.2	1.5±0.2	3.6±0.7	2.9±0.5	4.4±1.2	5.2±0.9

[a] Ash foliage was fed upon by 10, 8-10-d old EAB female adults for 48 h; * and ** denote significant difference at α=0.05 and 0.01 between control and EAB treatments within the same ash species, respectively

only in black ash; coumaroylquinic acid and feruloylquinic acid were detected only in green ash; and verbascoside hexoside was detected only in white ash (Tables 2, 3). EAB adult feeding did not elicit or decrease concentrations of any of a number of selected compounds (most prominent peaks in their respective chromatograms,,Table 3). The levels of total foliar phenolics in white ash decreased significantly upon EAB feeding but were unaffected in black and green ash foliage (Fig. 2).

EAB adult feeding did not affect production of trypsin inhibitor in any species (Fig. 3a). Neither was production of chymotrypsin inhibitor in green or white ash changed (Fig. 3b). Chymotrypsin inhibitor concentration of EAB adult-fed black ash foliage was greater than that of undamaged control (Fig. 3b).

Discussion

The most abundant constitutive volatiles from all three species were three green leaf volatiles [hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate] and a sesquiterpene (*Z,E*-α-farnesene). Indole was induced by EAB adult feeding in all three species. However, in previous work, indole did not elicit any electro-antennal activities of EAB females or males (Rodriguez-Saona et al. 2006).

Other volatiles detected from foliage responded differentially to EAB adult feeding. Headspace concentrations of hexanal and (*E*)-2-hexenal of black and white ash were not affected by EAB adult feeding, while they were suppressed in green ash. Both the reduction of these two compounds and the effect on EAB need further investigation because EAB males and females have been shown to be responsive to hexanal and (*E*)-2-hexenal in GC-EAD bioassays (Rodriguez-Saona et al. 2006; de Groot et al. 2008). However, neither of these two compounds seemed to be attractive to EAB males or females under field conditions (de Groot et al. 2008).

The monoterpene (*E*)-β-ocimene was among the most commonly released volatiles following herbivore damage. Its emission was not elevated in black ash, while it increased significantly in green and white ash. Many herbivores, including the sphinx moth *Manduca sexta* (Lepidoptera: Noctuidae) (Fraser et al. 2003) and EAB (Rodriguez-Saona et al. 2006) have been observed to respond to (*E*)-β-ocimene in EAD bioassays.

Elevated levels of four volatiles, (*Z,E*)-α-farnesene, α-humulene, β-caryophyllene, and cubenene, were induced by EAB feeding in white ash. These compounds have been suggested to be involved in host plant location by many herbivores and host location by some parasitoids (Dicke et al. 1990; Meiners et al. 2003). They have been found to be antennally-active in EAB (Rodriguez-Saona et al. 2006; Crook et al. 2008), and the latter three are components of *Manuka* oil which is attractive to EAB in the field (Crook et al. 2008).

Benzyl cyanide emission was elevated 6-fold by EAB feeding in black ash and 100-fold in white ash, while it was not affected in green ash. Benzyl cyanide is produced by both plants (Bartlet et al. 1997, Rodriguez-Saona et al. 2006) and insects (Seidelmann et al. 2000, Fatouros et al. 2008), and it has been hypothesized to have many ecological functions in intraspecific and interspecific interactions. For instance,

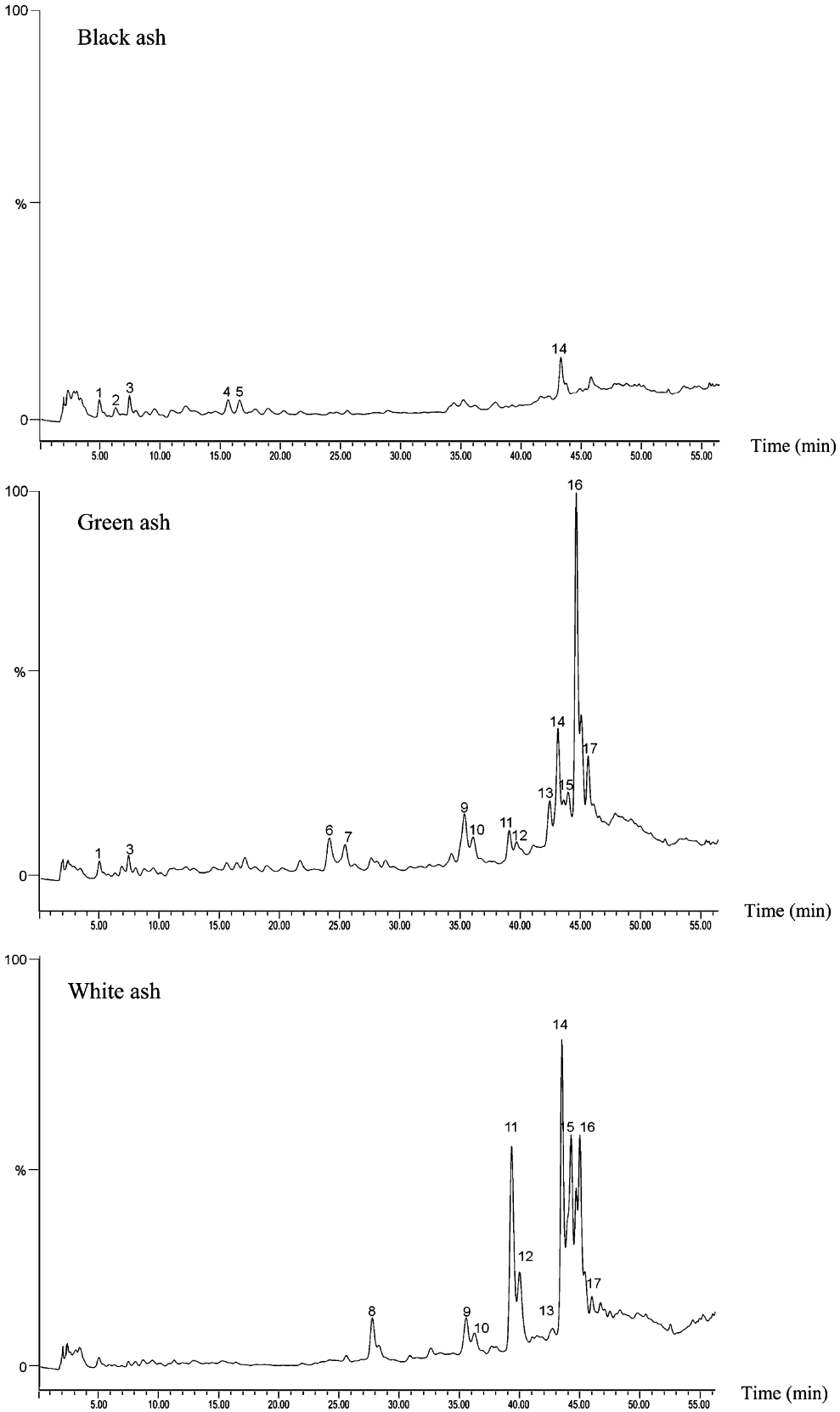


Fig. 1 HPLC-UV chromatographic profiles at 280 nm of methanol extracts of black, green, and white ash control foliage. Numbers above peaks refer to compounds listed in Table 2

Table 2 Tentative identification of phenolics from black, green and white ash based on retention time, *m/z*, MS fragmentation, and UV absorbance maxima

Peak Number	Species[a]	RT (min)	$[M-H]^-$ (*m/z*)	Fragments *m/z* (in order of decreasing abundance)	UV (λ) maxima (nm)	Putative ID	Reference
1	G, B	5.03	315	153, 123	278.9	Hydroxytyrosol hexoside	Cardoso et al. 2005
2	B	6.38	ND[b]	ND	360.3, 271.8	Unknown #1	N/A
3	G, B	7.51	299	113, 119, 143, 179	276.5	Tyrosol hexoside	Kammerer et al. 2005
4	B	15.82	ND	ND	312.1	Unknown #2	N/A
5	B	16.62	377	197, 153, 109	280.1, sh[c] 316	Oleuropein Aglycone	Cardoso et al. 2005
6	G	24.41	337	191, 163, 173, 127, 85	sh 290, 312.1	Coumaroylquinic Acid	Poon 1998
7	G	25.61	367	ND	280.1, 333.5	Feruloylquinic Acid	Bastos et al. 2007
8	W	27.79	785	623, 461, 315	sh 285, 331.1	Verbascoside hexoside	Eyles et al. 2007
9	G, W	35.52	623	461, 315, 135	sh 285, 331.1	Verbascoside	Cardoso et al. 2005
10	G, W	36.13	685	523, 453, 421, 299, 153, 179	280.1, 331.1	Nuzhenide	Ryan et al. 2002
11	G, W	39.29	569	537, 403, 223, 357,121	281.2	Oleuropein related compound	Cardoso et al. 2005, Savarese et al. 2007
12	G, W	39.77	539	377, 307, 275, 139	281.2	Oleuropein	Tanahashi et al. 1998
13	G, W	42.62	609	301, 271, 179, 151	265.9, 349.0	Rutin	Savarese et al. 2007
14	G, W, B	43.35	523	361, 291, 259, 111, 139	sh 280	Ligustroside	Ryan et al. 2002
15	G, W	44.23	577	269, 225, 197	267.0, 339.5	Apigenin-7-*O*-rutinoside	Plazonić et al. 2009
16	G, W	44.86	593	285, 255, 211, 227	268.2, sh 290, 338.3	Kaempferol-O-coumaroyl hexoside	Gouveia and Castilho 2010
17	G, W	45.89	593	285, 257	265.9, sh 290, 350.2	Luteolin-7-*O*-rutinoside	Plazonić et al. 2009

[a] B: black ash; G: green ash; W: white ash

[b] ND = Not detected

[c] Shoulder

benzyl cyanide released from oilseed rape (*Brassica napus* L.) serves as an attractant for cabbage seed weevil *Ceutorhynchus assimilis* (Coleoptera: Curculionidae) (Bartlet et al. 1997). In insects, male-originated benzyl cyanide functions as an anti-aphrodisiac for females and kairomones for *Trichogramma brassicae* (Hymenoptera:Trichogrammatidae), the egg parasitoid of *P. brassicae* females (Fatouros et al. 2005). However, benzyl cyanide was not among the volatile compounds in Manchurian ash that elicited electroantennal activity in EAB adult males or females (Rodriguez-Saona et al. 2006).

Three noteworthy points concerning volatile ash emissions in this study are: (1) A lack of antennal or behavioral response by EAB to significantly induced volatiles such as indole does not necessarily negate an effect on other organisms such as predators and parasitoids of EAB. Attraction of herbivore-induced plant volatiles to natural enemies of herbivores is well documented (Heil 2008; Chen et al. 2010); (2) Although not significantly elevated, levels of (*Z,E*)-α-farnesene, α-humulene, β-caryophyllene, and cubenene in EAB fed seedlings of green and black ash were numerically higher than those in respective control seedlings. The effects of potential increases of these compounds on attraction of EAB requires further investigation; (3) The effects of changes in ratios of these compounds deserve further examination because not only trap placement, trap size and color, but also ratios and release rates of VOCs, are important in trapping and detection of EAB (Crook and Mastro 2010).

Table 3 Concentrations (mean±1SE mg g^{-1} fresh weight) of selected individual phenolic compounds in green, white, and black ash

Peak #[a]	Black ash		Green ash		White ash	
	Constitutive (*N*=8)	Induced[a] (*N*=8)	Constitutive (*N*=8)	Induced (*N*=8)	Constitutive (*N*=7)	Induced (*N*=7)
1	$6.5\times10^{-2}\pm1.4\times10^{-2}$	$5.9\times10^{-2}\pm1.3\times10^{-2}$	$6.4\times10^{-2}\pm0.6\times10^{-2}$	$5.7\times10^{-2}\pm0.7\times10^{-2}$	ND	ND
3	$4.6\times10^{-2}\pm0.3\times10^{-2}$	$5.3\times10^{-2}\pm0.8\times10^{-2}$	$0.5\times10^{-1}\pm0.1\times10^{-1}$	$3.2\times10^{-2}\pm0.4\times10^{-2}$	ND	ND
5	$25.6\times10^{-2}\pm5.2\times10^{-2}$	$40.5\times10^{-2}\pm13.5\times10^{-2}$	ND	ND	ND	ND
8	ND[b]	ND	ND	ND	$26.8\times10^{-2}\pm6.1\times10^{-2}$	$12.8\times10^{-2}\pm2.5\times10^{-2}$
9	ND	ND	$49.1\times10^{-2}\pm12.7\times10^{-2}$	$5.7\times10^{-1}\pm1.6\times10^{-1}$	$47.5\times10^{-2}\pm9.0\times10^{-2}$	$50.9\times10^{-2}\pm2.8\times10^{-2}$
10	ND	ND	$58.7\times10^{-2}\pm1.6$	$3.2\times10^{-1}\pm.18\times10^{-1}$	$74.3\times10^{-2}\pm12.8\times10^{-2}$	70×10^{-2} 3±8.3$\times10^{-2}$
11	ND	ND	$62.2\times10^{-2}\pm16.5\times10^{-2}$	$39.5\times10^{-2}\pm2.2\times10^{-2}$	4.6±1.1	2.5±0.9
12	ND	ND	$38.9\times10^{-2}\pm9.5\times10^{-2}$	$40.6\times10^{-2}\pm7.8\times10^{-1}$	3.6±0.8	1.8±0.7
13	ND	ND	$32.4\times10^{-2}\pm5.1\times10^{-2}$	$40.4\times10^{-2}\pm7.7\times10^{-2}$	$13.5\times10^{-2}\pm4.5\times10^{-2}$	$13.8\times10^{-2}\pm6.5\times10^{-2}$
14	0.6±0.1	0.4	1.6±0.5	0.3±0.3	4.4±1.7	2.0±0.5
15	ND	ND	$8.3\times10^{-2}\pm3.6\times10^{-2}$	$33.5\times10^{-2}\pm3.0\times10^{-2}$	$39.7\times10^{-2}\pm10.9\times10^{-2}$	$27.6\times10^{-2}\pm0.2\times10^{-2}$
16	ND	ND	$51.1\times10^{-2}\pm7.7\times10^{-2}$	$75.5\times10^{-2}\pm15.7\times10^{-2}$	$37.1\times10^{-2}\pm7.7\times10^{-2}$	$22.3\times10^{-2}\pm1.9\times10^{-2}$
17	ND	ND	$21.4\times10^{-2}\pm6.2\times10^{-2}$	$7.3\times10^{-2}\pm1.8\times10^{-2}$	$7.9\times10^{-2}\pm2.9\times10^{-2}$	$7.8\times10^{-2}\pm4.8\times10^{-2}$

No significant differences for each individual phenolic compound were found from the same ash species between control and induced foliage

[a] See Table 2 for tentative identification; Ash foliage was fed upon by 10, 8-10-d old EAB female adults for 48 h

[b] ND=Not detected

Phenolics generally denature plant proteins and thus reduce nutritive values of plants (Dudt and Shure 1994; Ossipov et al. 2001). Phenolics also can inhibit the absorption of key amino acids. For example, cyclochampedol, a flavonoid from acetone bark extracts of the tree *Arctocarpus champeden* (Thunb.) Merr., inhibits absorption of leucine in the midgut of the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) (Parenti et al. 1998). Significant qualitative differences among the three ash species were observed. Qualitative difference of phloem phenolics between the Asian ash species, Manchurian ash, and two North American species, green and white ash, have been noted previously (Eyles et al. 2007). Our study shows also, however, that compared to phloem, ash foliage appears to contain fewer phenolic compounds, regardless of ash species.

EAB adult feeding did not induce accumulation of individual selected foliar phenolics in the present study, irrespective of species, perhaps due to the nutrient regime used. For example, many volatile and non-volatile compounds that are involved in cotton (*Gossypium hirsutum* cv. FiberMax 989) or Austrian pine (*Pinus nigra* Arnold) defense are suppressed by high nitrogen fertilization (Blodgett et al. 2005; Chen et al. 2008), and the nitrogen supply in the present study was relatively high. Second, the lack of induction likely was due to a phenomenon called "delayed induced resistance" (DIR) in which induction of defensive compounds occurs in the years following defoliation. Induced resistance can be either DIR or "rapidly induced resistance" (RIR) in which defensive compounds appear shortly after damage (Karban and Myers 1989). DIR has been observed in many plant species, in particular, in trees (Kaitaniemi et al. 1998). Third, the lack of induction might reflect a general pattern of the response of mature foliage to biotic and abiotic stress (Nichole-Orians 1991; McAuslane et al. 1997; Chen and Poland 2009b).

However, EAB adult feeding induced a decrease of total soluble phenolics in white ash (Fig. 1). This may partially explain why EAB adults consumed more white ash foliage given choices between black and white ash foliage (Chen and Poland 2010). Furthermore, the

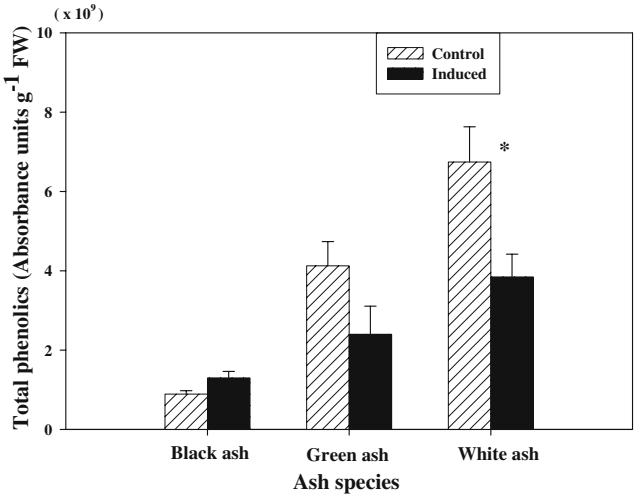


Fig. 2 Total soluble phenolics (mean±SE absorbance units g^{-1} fresh weight tissue) in control (undamaged) foliage and foliage induced by emerald ash borer (EAB) adult feeding of black, green, and white ash. Data were analyzed by independent *t*-test, separately for ash species. Significant differences within species are indicated by asterisks (*: $P<0.05$). Black ash: $N_{Control}=8$; $N_{EAB}=8$; Green ash: $N_{Control}=8$; $N_{EAB}=8$; White ash: $N_{Control}=7$; $N_{EAB}=7$

longevity of EAB adults was greater when fed white ash compared to black ash (Chen and Poland 2010). The three phenolics uniquely detected in black ash (oleuropein aglycone, unknowns #1 and 2; Table 2) might also cause EAB adults to prefer green and white ash over black ash (Chen and Poland 2010). Compared to green and white ash, black ash is phylogenetically more closely related to Manchurian ash (Wallander 2008), which has been observed to be more resistant to EAB infestation (Rebek et al. 2008).

Many protease inhibitors are involved in herbivore defense (Ryan 1990, Zavala et al. 2004). Protease inhibitors impair herbivore growth by eventual depletion of essential amino acids resulting from a herbivore's compensatory production of digestive proteases (Broadway and Duffey 1986). EAB feeding did not affect trypsin inhibitors of black, green, or white ash (Fig. 3a), or chymotrypsin inhibitors of green or white ash (Fig. 3b). The activity of chymotrypsin inhibitors, however, did increase in black ash as a result of EAB feeding (Fig. 3b), which, together with the unique phenolics in black ash discussed above, might contribute to the preference of EAB adults for green and white ash over black ash (Chen and Poland 2010).

In summary, EAB adult feeding differentially affected volatile and non-volatile compounds of the three most common ash species in northeastern USA. Black ash was the least responsive species in terms of volatile induction with only two (indole and benzyl cyanide) of eleven compounds responding. Green ash was more responsive than black ash, with two [(*E*)-β-ocimene and indole] of eleven volatiles induced while two green leaf volatiles [hexanal and (*E*)-2-hexenal] decreased. White ash showed the strongest response with an increase in seven of eleven compounds. Black and white ash seedlings were more responsive to EAB adult feeding than green ash in terms of two non-volatile, putatively defensive compound groups, i.e., phenolics and protease inhibitors. Phenolic production decreased in white ash while chymotrypsin increased in black ash. The induction of chymotrypsin inhibitors by EAB feeding, together with unique phenolics in black ash, might explain, in part, EAB adults' preference for green and white ash over black ash (Chen and Poland 2010). Future studies will focus on screening of potent compounds from ash foliar volatile and non-volatile compounds identified in this study and incorporating them into ash breeding programs to develop resistant trees.

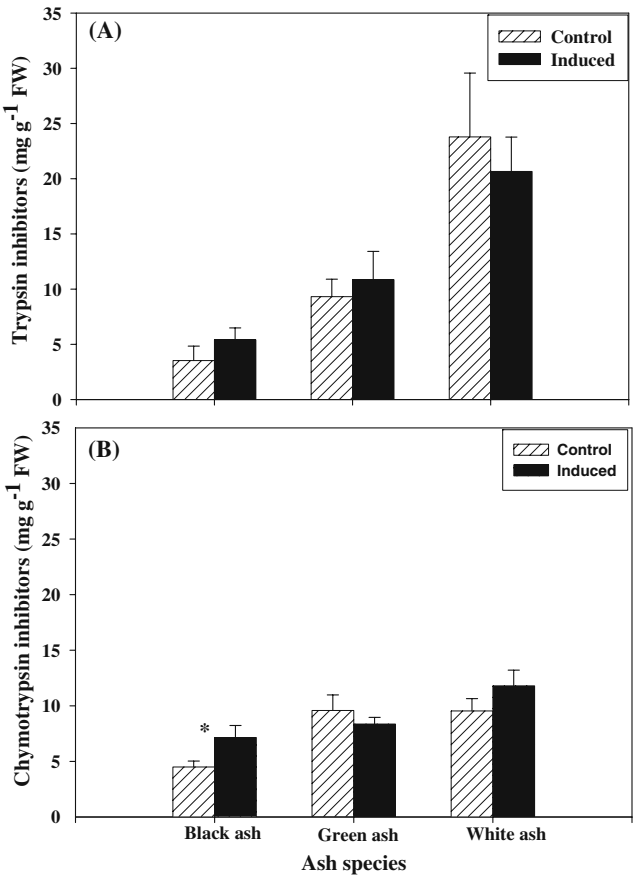


Fig. 3 Total concentrations (mean±1SE mg g^{-1} fresh weight tissue) of foliar trypsin **a** and chymotrypsin inhibitors **b** in control (undamaged) foliage and foliage induced by emerald ash borer (EAB) adult feeding of black, green, and white ash. Data were analyzed by independent *t*-test, separately for ash species. Significant differences within species are indicated by asterisks (*: $P<0.05$). Black ash: $N_{Control}=8$; $N_{EAB}=8$; Green ash: $N_{Control}=8$; $N_{EAB}=8$; White ash: $N_{Control}=8$; $N_{EAB}=8$

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Behavioral Responses of the Leafhopper, *Cicadulina storeyi* China, a Major Vector of Maize Streak Virus, to Volatile Cues from Intact and Leafhopper-Damaged Maize

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Abstract The chemical ecology of the leafhopper, *Cicadulina storeyi* China (Homoptera: Cicadellidae), an important vector of Maize Streak Virus (MSV), was studied with a view to developing novel leafhopper control strategies in sub-Saharan Africa. Choice tests using a Y-tube olfactometer revealed that odors from uninfested maize seedlings (*Zea mays* cv. Delprim) were significantly more attractive to *C. storeyi* than odors from *C. storeyi*-infested seedlings. Headspace samples of volatile organic compounds (VOCs) collected from 10 to 12 day-old uninfested seedlings were more attractive than those collected from infested seedlings. While VOCs collected from uninfested maize seedlings were attractive, VOCs collected from C. *storeyi*-infested seedlings were significantly repellent. Analysis of the collected VOCs by gas chromatography (GC) and coupled GC-mass spectrometry (GC-MS) led to the identification of myrcene, linalool, (*E*)-2-decen-1-ol, and decanal from uninfested seedlings, and (*Z*)-3-hexenyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), methyl salicylate, benzyl acetate, indole, geranyl acetate, (*E*)-caryophyllene, α-bergamotene, (*E*)-β-farnesene, β-sesquiphellandrene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) from infested seedlings. Of these, methyl salicylate, (*E*)-caryophyllene, (*E*)-β-farnesene, and TMTT were identified previously as volatile semiochemicals involved in plant defense against other sucking insect pests. When tested individually for behavioral activity, all compounds were repellent for *C. storeyi*. Moreover, when these induced VOCs were added to the blend of VOCs from uninfested maize seedlings, a shift from attraction to repellency was observed. Addition of methyl salicylate, (*E*)-β-farnesene, or TMTT resulted in a choice for the solvent control (i.e., repulsion), whereas addition of (*E*)-caryophyllene resulted in no reduction in host VOC attractiveness. These results show that VOCs induced in maize have the potential to be exploited in the control of viruliferous leafhoppers in sub-Saharan Africa.

Key Words Maize · *Zea mays* · Leafhoppers · *Cicadulina storeyi* · Volatile organic compounds · Methyl salicylate · Sesquiterpenes · Y-tube olfactometer · Repellency

Introduction

Maize, *Zea mays* L. (Poaceae), is the third major cereal crop in the world after wheat and rice, and is the most important crop in sub-Saharan Africa, where it has gradually displaced native food crops such as sorghum and millet, and is grown for human consumption, animal feed, and several industrial uses. The greatest constraints to higher yields are pests, diseases, and parasitic weeds (Efron et al., 1989). Maize can supply 50% of the caloric intake in sub-Saharan Africa, but in certain years, a farmer's entire crop can be wiped out by Maize Streak Virus (MSV) (Geminiviridae: genus *Mastrevirus*), an endemic pathogen of native African grasses, which is the most important virus disease of maize in sub-Saharan Africa (Thottappilly et al., 1993). It is acquired and

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transmitted in a persistent manner by *Cicadulina* spp. leafhoppers (Homoptera: Cicadellidae), although the ability to transmit MSV varies among species. A vector insect can retain and transmit the virus throughout its lifespan. There are five *Cicadulina* species, *C. storeyi, C. mbila, C. arachidis, C. ghaurii*, and *C. dabrowskii*, associated with MSV transmission in Nigeria, the two former species being the most efficient vectors (Asanzi et al., 1995; Oluwafemi et al., 2007). The timing of infection, the maize genotype, and prevailing climatic conditions can all influence the extent of damage, which is often economically significant. In Nigeria, 100% infection has been recorded in some fields (Fajemisin et al., 1976), while outbreaks of the disease, causing economic losses, have been reported in over 20 countries (Kim et al., 1981).

Control of MSV disease has been based on cultural practices, including time of planting and rotation of maize crops, the use of chemical insecticides incorporated in the soil at planting, and the use of resistant varieties. However, these practices do not control the leafhopper vectors, since both virus and vectors have wide alternative host ranges in wild grasses around maize fields, and survive on wild grasses during off seasons when maize is not cultivated. In addition, the gradual increase in the use of irrigation in African agriculture has extended the growing period for maize, which in turn has increased vector populations and the source of virus inoculums. Chemical insecticides are expensive for resource-poor farmers, and are perceived as having a negative environmental impact. Breeding of maize for durable resistance to MSV has been a long-term research strategy for protection of maize crops (Fajemisin et al., 1984). Quantitative Trait Loci (QTLs) for maize resistance to MSV have been mapped (Pernet et al., 1999), and an MSV-resistant transgenic maize has been developed (Shepherd et al., 2007). However, such technology is unlikely to be available for the poorest of sub-Saharan maize farmers. Taken together, barriers to the deployment of insecticide and genetic-based strategies for MSV control make the search for alternative strategies, such as exploitation of naturally-occurring plant defense processes, urgent.

One possibility is to exploit volatile semiochemicals (naturally-occurring behavior and development-modifying chemicals) released by plants following insect herbivory, which can deter herbivore colonization, while at the same time attracting insects antagonistic to herbivores, such as parasitoids and predators (Dicke et al., 1993, 2003; Karban and Baldwin, 1997). Knowledge of the induced plant defense chemistry in maize upon leafhopper damage could lead to the selection and/or development of varieties with more durable resistance, through enhanced constitutive or induced plant defense (Bernasconi et al., 1998; Stout and Davis, 2009).

This study provides the first examination of the chemical ecology of the interaction between maize and *C. storeyi*, specifically an investigation of the behavioral responses of *C. storeyi* to volatile organic compounds (VOCs) released by maize seedlings when infested by *C. storeyi,* and the response to such VOCs in laboratory bioassays. Our findings provide the first steps in developing novel control options that exploit naturally occurring processes by which maize plants resist damage by leafhoppers.

Methods and Materials

Insects and Plant Material *Cicadulina storeyi* China (Homoptera: Cicadellidae) leafhoppers, free of Maize Streak Virus (MSV), were collected from the mass rearing colony of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, and reared on pearl millet, *Pennisetum americanum* (= *typhoides*) in a quarantine growth facility (23±2°C, 40% RH, 16:8 h, L:D) at Rothamsted Research, Harpenden, Hertfordshire, UK. The leafhoppers and host plants were placed in plexi-glass cages (44×44×70 cm) with two netted openings (12×12 cm) in the front while a third netted opening (11×11 cm) at the back was fitted with an electric fan for air circulation. Maize, *Zea mays* (cv. Delprim) seeds were bought from Delley Samen und Pflanzen AG, Delley Semences et Plantes SA, Le Chateau, Switzerland, and grown under controlled conditions at Rothamsted (25±1°C; 16:8 h, L:D; 150 $\mu E.m^{-2}.s^{-1}$).

Y-Tube Olfactometer Bioassay Bioassays were conducted in a controlled environment room (22±1°C, 40% Relative Humidity) fitted with an extraction fan. A glass Y-tube olfactometer (2 cm internal diam., 16 cm stem length, 14 cm arms length) was set up in a black cage (60×60×76 cm) consisting of a steel frame covered with black cardboard paper to avoid external visual cues. The olfactometer was clamped onto a tripod in an inclined position (70° from the horizontal plane). Uniform illumination was provided by two fluorescent light tubes (70 W; Luminux) positioned approximately 25 cm above the Y-tube junction. A charcoal filtered airstream (200 ml min^{-1}) was supplied to each arm of the olfactometer by using an electric pump. Each airstream then passed through a glass jar containing test material (either live uninfested or *C. storeyi*-infested maize seedlings, or a piece of filter paper dosed with 1 µl of VOC sample or solution of synthetic chemical). *C. storeyi* were collected from rearing cages in a separate insectary room and starved for 2 hr before each trial. Ten adult *C. storeyi* were used in each replicate, and the experiments were replicated ten times. After each replicate, the olfactometer was rotated 180° to avoid directional effects. The numbers

of leafhoppers entering each arm as a final choice were recorded. A leafhopper was deemed to have made a final choice if it entered an arm but did not return back within 10 min to enter the opposite arm. Bioassays were conducted throughout the day, but with treatments alternated, to avoid any effect of time of day on response. No effects were observed regarding the effect of time of day on *C. storeyi* behavior.

Four series of dual-choice test bioassay experiments were carried out. In Series 1, *C. storeyi* were directly exposed to odors ducted from single maize seedlings. Responses to odors from uninfested and *C. storeyi*–infested maize seedlings were compared, the latter of which were prepared by infestation with 50 leafhoppers for a period of 24 hr prior to use. In Series 2, responses to VOCs collected from seedlings were compared as follows: (i) uninfested VOCs vs. diethyl ether solvent; (ii) *C. storeyi*—infested VOCs vs. diethyl ether solvent; and (iii) uninfested VOCs vs. *C. storeyi*—infested VOCs. In Series 3, responses to synthetic compounds (1 μg in 1 μl hexane) identified as being induced by *C. storeyi* were tested against the solvent control. Finally, in Series 4, responses to a headspace sample of uninfested maize VOCs alone and in combination with 1 μg doses of synthetic equivalents of *C. storeyi*-induced compounds were compared to a solvent control. The dose of headspace sample (from clean and infested maize) used per bioassay was equivalent to the VOCs emitted by one maize seedling / hr.

Statistical Analysis The Y-tube olfactometer data were analyzed using a paired *t*-test with statistical software (GenStat Version 10, VSN International Ltd.) after ensuring that data were normally distributed.

Volatile Organic Compound (VOC) Collection Air entrainment (also referred to as dynamic headspace collection) was carried out following standard procedures (Agelopoulos et al. 1999) using 10 to 12 day-old maize seedlings. Seeds were pre-germinated and then planted singly in 13 cm (5″) plastic pots inside a growth chamber (25°C, 16:8 h, L:D). For each collection, a single seedling was enclosed in a glass vessel (22 cm high × 6.5 cm internal diam) open at the bottom with two collection ports at the top (one for inlet and the other for outlet). The bottom was closed without pressure around the plant stem by using two semicircular aluminium plates with a hole in the center to accommodate the stem. The plates were clipped to a flange on the open end of the glass vessel. Air, purified by passage through an activated charcoal filter, was pumped into the vessel through the inlet port at 700 ml min^{-1} (flow rate controlled by a needle valve and measured by a flow meter). Air was drawn out at 600 ml min^{-1} through Porapak Q (50 mgs) in a 5-mm diam glass tube (Alltech Associates, Camforth, Lancashire, UK). The difference in flow rates created a slight positive pressure to ensure that unfiltered air did not enter the system, thus removing the need for an airtight seal around the stem. All connections were made with polytetrafluoroethylene (PTFE) tubing (Alltech Associates) with brass ferrules and fittings (North London Valve, London, UK) and sealed with PTFE tape (Gibbs & Dany, Luton, UK). Glassware, metal plates, and other equipment were washed with Teepol detergent (Herts County Supplies, Herts, UK) in an aqueous solution, acetone, and distilled water, and then baked overnight at 180°C. Porapak Q tubes were conditioned before use by washing with redistilled diethyl ether (1 ml) and heated to 132°C for 2 hr under a stream of constant nitrogen. Charcoal filters (BDH, 10–14 mesh, 50 g) were conditioned before use by purging with nitrogen inside an oven (150°C) for a minimum of 2 hr. Once conditioned, filters were sealed using Swagelok cap fittings and left to cool to ambient temperature prior to use. VOCs collected on Porapak Q were eluted with freshly distilled diethyl ether (750 μl). Eluted samples were then concentrated to *ca.* 100 μl under a gentle stream of nitrogen and stored in tightly capped microvials in a freezer (−20°C) until required for use. Plants were entrained for 7 d, but the Porapak Q tubes were changed daily, ensuring 24 hr entrainment periods. VOCs were collected from clean and *C. storeyi*—infested maize seedlings. For the latter, seedlings were infested with 50 adults per plant, and allowed to settle for 1 hr prior to commencement of VOC collection. For behavior assays and chemical analysis, VOC samples collected during 24–48 hr, 48–72 hr, and 72–96 hr were pooled together and concentrated to ca. 100 μl, to provide headspace samples equivalent to VOCs emitted by one maize seedling / hr. 0–24 Hr samples were excluded as behavior assays with live plants were conducted 24 hr after infestation, and samples collected beyond 96 hr were excluded to avoid possible interference from stress caused by placement of plants in volatile collection chambers. Entrainments from clean and infested plants were replicated 3 times.

Gas Chromatography (GC) Analysis VOCs were analyzed on a Hewlett-Packard 6890 GC equipped with a cool-on-column injector, a flame ionization detector (FID), and a non-polar HP-1 bonded phase fused silica capillary column (50 m×0.32 mm i.d., film thickness 0.52 μm). The oven temperature was maintained at 30°C for 1 min, and programmed at 5°C min^{-1} to 150°C and held for 0.1 min, then 10°C min^{-1} to 230°C. The carrier gas was hydrogen. Four μl of each eluted sample were injected into the injector port of the GC instrument. Data were analyzed using HP Chemstation software.

Coupled GC-Mass Spectrometry (GC-MS) GC-MS analysis of collected VOCs was performed using a fused silica capillary column (50 m×0.32 mm i.d., film thickness 0.52 μm, DB-1), attached to a cool on-column injector, which was directly coupled to a magnetic sector mass spectrometer (Autospec Ultima, Fisons Instruments, Manchester, UK). Ionization was by electron impact (70 eV, source temperature 250°C). Helium was the carrier gas. The oven temperature was maintained at 30°C for 5 min, and then programmed at 5°C min^{-1} to 250°C. Tentative identifications were made by comparison of spectra with mass spectral databases (NIST, 2005). Identifications were confirmed by peak enhancement on GC using authentic compounds (Pickett, 1990).

Chemicals Porapak Q (80/100 mesh) was purchased from Alltech (PA, USA). Diethyl ether (purris.) was purchased from Sigma Aldrich. Hexane (95% pesticide residue) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK) and distilled prior to use. Methyl salicylate and (*E*)-caryophyllene were purchased from Sigma Aldrich (Steinheim, Germany). (*E*)-β-Farnesene (>98%) was synthesized from (*E*)-farnesyl chloride (Kang et al., 1987). (*E*, *E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT) was synthesized from (*E,E*)-farnesol, by oxidation to its corresponding aldehyde (*E,E*)-farnesal, followed by Wittig methylenation (Leopold, 1990).

Results

Bioassay Series 1—Responses to Odors from Uninfested and Infested Maize Seedlings In dual-choice Y-tube olfactometer bioassays, *C. storeyi* preferred the odor from uninfested maize seedlings to the odor from *C. storeyi* infested seedlings (P=0.018) (Fig. 1).

Bioassay Series 2—Responses to VOC Samples VOCs collected from uninfested maize were attractive (P=0.003), while VOCs from *C. storeyi*-infested maize were repellent (P=0.021) (Fig. 2). In a dual-choice experiment, *C. storeyi* preferred VOCs from uninfested maize seedlings compared to VOCs from *C. storeyi*-infested seedlings (P<0.001) (Fig. 2).

VOC Analysis The VOC profile of 10 to 12 day-old maize seedlings was altered markedly when plants were infested with *C. storeyi*. Analysis of the odor blends, which were collected in 24–48 hr, 48–72 hr, and 72–96 hr periods and combined, using GC and coupled GC-MS (Fig. 3), showed that myrcene, linalool, and decanol were produced by both uninfested and infested plants. However, seedlings infested with *C. storeyi* additionally produced (*Z*)-3-hexenyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), benzyl acetate, methyl salicylate, indole, geranyl acetate, (*E*)-caryophyllene, α-bergamotene, (*E*)-β-farnesene, β-sesquiphellandene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). The identity of methyl salicylate, (*E*)-caryophyllene, (*E*)-β-farnesene, and TMTT was confirmed by GC peak enhancement using authentic samples. Replication (N=3) of VOC collection over the same 7 d period confirmed consistent emissions from clean and infested seedlings (data not shown).

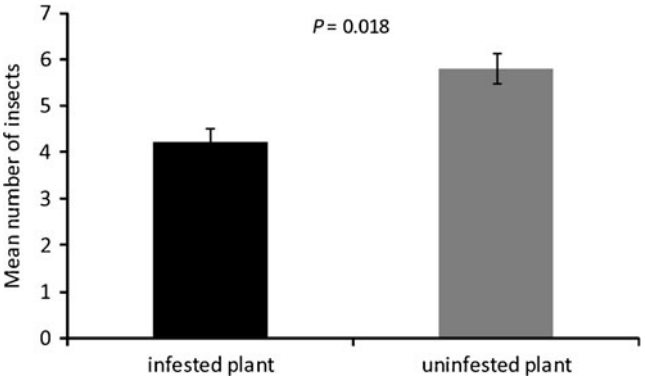


Fig. 1 Mean (± standard error) number of leafhoppers, *Cicadulina storeyi*, choosing odors from infested and uninfested maize seedlings in a Y-tube olfactometer. Statistics: *t*-test; N=10 replicates each with 10 individuals

Bioassay Series 3—Responses to Synthetic VOCs *C. storeyi* preferred the solvent control over methyl salicylate (P=0.002) (Fig. 4). Similarly, the induced VOCs (*E*)-β-farnesene, (*E*)-caryophyllene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) were repellent compared to the solvent control (P=0.034, P=0.043 and P=0.048, respectively) (Fig. 4).

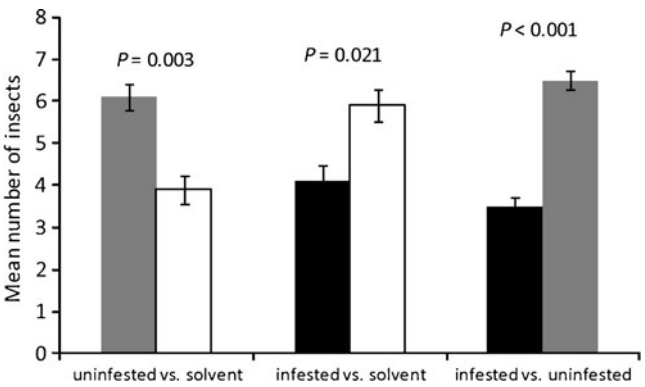


Fig. 2 Mean (± standard error) number of leafhoppers, *Cicadulina storeyi*, choosing VOCs or solvent control in a Y-tube olfactometer. Statistics: *t*-test; N=10 replicates each with 10 individuals

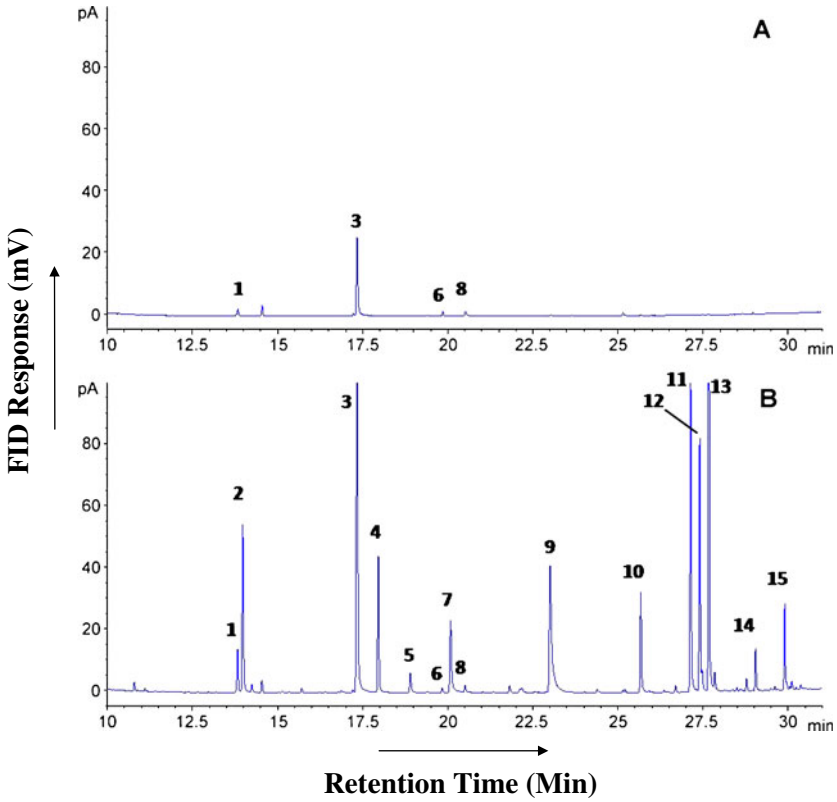


Fig. 3 Gas chromatograms of VOCs emitted by maize seedlings (cv. Delprim) 2–4 d after infestation. A. Uninfested maize seedlings. B. Maize seedlings infested with *Cicadulina storeyi* Key: 1. Myrcene 2. (*Z*)-3-Hexenyl acetate 3. Linalool 4. (*E*)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) 5. Benzyl acetate 6. (*E*)-2-Decen-1-ol 7. Methyl salicylate 8. Decanal 9. Indole 10. Geranyl acetate 11. (*E*)-Caryophyllene 12. α-Bergamotene 13. (*E*)-β-Farnesene 14. β-Sesquiphellandrene 15. (*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT). Compound identifications remain tentative apart from 7, 11, 13 and 15, which were confirmed by GC peak enhancement using authentic samples

Bioassay Series 4—Responses to C. storeyi-Inducible Compounds in the Presence of VOCs from Uninfested Maize When methyl salicylate was added to VOCs collected from uninfested maize, more *C. storeyi* preferred the solvent control ($P<0.001$) (Fig. 5). Addition of (*E*)-β-farnesene and TMTT to VOCs from uninfested maize also resulted in preference for the solvent control ($P=0.003$ and $P=0.048$, respectively), whereas *C. storeyi* still preferred VOCs from uninfested maize despite the addition of (*E*)-caryophyllene ($P=0.01$) (Fig. 5).

Discussion

Analysis of the VOCs collected from maize seedlings (cv. Delprim) infested with *C. storeyi* by gas chromatography

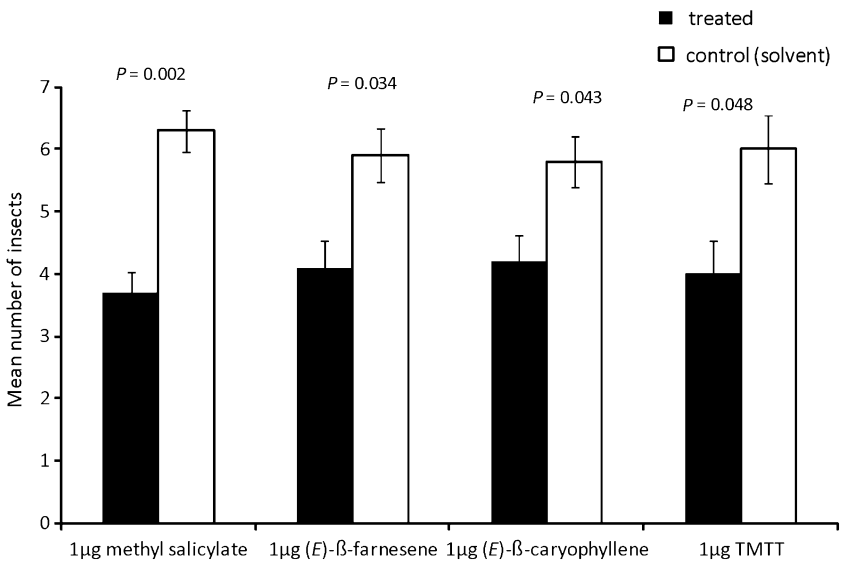


Fig. 4 Mean (± standard error) number of leafhoppers, *Cicadulina storeyi*, choosing synthetic induced VOCs or solvent control in a Y-tube olfactometer. Statistics: *t*-test; $N=10$ replicates each with 10 individuals

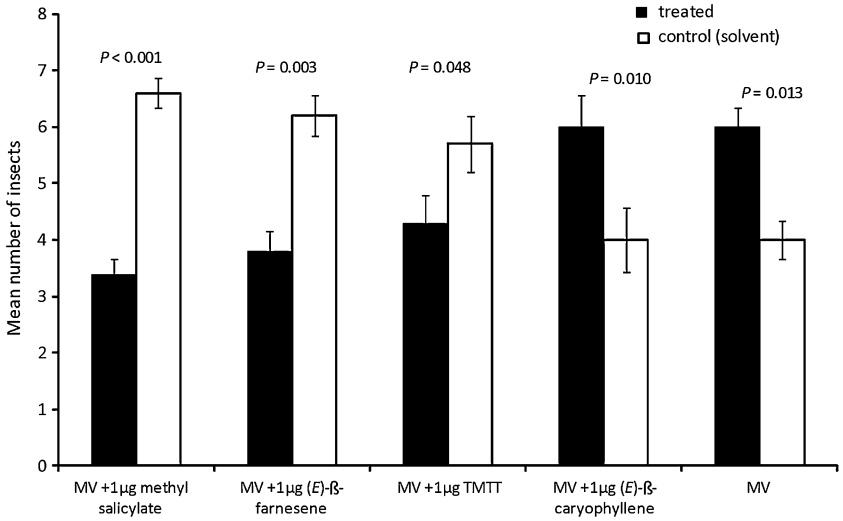


Fig. 5 Mean (± standard error) number of leafhoppers, *Cicadulina storeyi*, choosing maize VOCs (MV) in combination with synthetic induced VOCs or solvent control (indicated by MV only) in a Y-tube olfactometer. Statistics: *t*-test; *N*=10 replicates each with 10 individuals

(GC) and coupled GC-mass spectrometry (GC-MS) showed that the VOC profile was similar to those induced by the leafhopper, *Euscelidius variegatus* (Erb, 2009), and *Spodoptera* spp. caterpillers (*S. littoralis* and *S. exigua*) (Hoballah et al., 2002, 2004; Hoballah and Turlings, 2005; Gouinguene et al., 2003, 2005; Farag et al., 2005; Schmelz et al., 2003). Tentative identifications included myrcene, linalool, (*E*)-2-decen-1-ol, and decanal from uninfested seedlings, and (*Z*)-3-hexenyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), methyl salicylate, benzyl acetate, indole, geranyl acetate, (*E*)-caryophyllene, α-bergamotene, (*E*)-β-farnesene, β-sesquiphellandrene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) from infested seedlings. Replication of volatile collections from clean and infested plants confirmed a consistent pattern in VOC profiles. Although samples collected beyond 96 hr were excluded to avoid possible interference from stress caused by placement of plants in volatile collection chambers, no visible sign in the production of stress cues was observed (data not shown). The strong induced emission of methyl salicylate following *C. storeyi* infestation contrasts with a previous study with *E. variegatus* (Erb, 2009), where no methyl salicylate was reported. This difference cannot be attributed to differences in feeding behavior, as both *C. storeyi* and *E. variegatus* are known to be phloem-feeders, but instead could be due to the fact that a longer duration of VOC collection was used in our study, compared to that of Erb (2009). It is possible that this strong induction of emission of methyl salicylate contributes to systemic immunity against MSV infection, as the compound has been demonstrated to act as a critical mobile signal in plants to mediate systemic immunity against viral and bacterial diseases (Park et al., 2007; Vlot et al., 2008).

The induction of plant defense in maize by leafhoppers appears to be much weaker on a per individual basis compared to caterpillars. In our study, five adult leafhoppers were used per maize seedling in preliminary experiments (data not shown), and an increase in insect density, to 50 individuals per seedling, was required in order to achieve detectable amounts of VOCs. Erb (2009) also used 40 adults of *E. variegatus* per maize seedling to achieve emission of a quantity of volatiles similar to 3 sec instars of *S. littoralis*. This was attributed to the lower amounts of damage inflicted by the stylets of *E. variegatus*, compared to the chewing mouthparts of *S. littoralis*. Furthermore, previous studies in our laboratory have shown that there is diel periodicity in the production and release of VOCs by maize (Chamberlain et al., 2006). Thus, future work will investigate the dynamics of VOC release in maize upon leafhopper infestation.

VOC production in maize has been shown to be induced by elicitors that originate from herbivores, e.g., volicitin, which was originally isolated and identified from the regurgitant of *S. exigua* (Alborn et al., 1997; Frey et al., 2000), and caeliferins, which were isolated and identified from the American bird grasshopper, *Schistocerca americana* (Alborn et al., 2007). VOC production is linked to transcription of specific genes, e.g., terpene synthase genes for the production of sesquiterpenes, DMNT and TMTT, of which several have been isolated and characterised (Degenhardt et al., 2009 and refs therein), and *IGL* that encodes indole-3-glycerol phosphate lyase for the production of indole (Frey et al., 2000). Production of methyl salicylate in tobacco is under the control of salicylate methyltransferase (SAMT) (Ross et al., 1999). Interestingly, salicylate methyltransferase activity in *Arabidopsis* is controlled by the *AtBSMT1* gene, for which transcription induction is strongly responsive to infestation by larvae of *Plutella xylostella*, physical wounding, and treatment with methyl jasmonate (Chen et al., 2003). Although a herbivore-inducible salicylate methyltransferase gene homologue has been cloned from maize (Lawrence and Novak, 2004), it has not been demonstrated that this gene is actually responsible for emission of methyl salicylate. The

results in our study suggest the production of an elicitor of plant defense by *C. storeyi*, which is capable of inducing defense genes as for other elicitors. Further work is underway to investigate the induction of mRNA transcripts for defense genes following *C. storeyi* infestation, and to characterize the elicitor of plant defense from this major plant disease vector.

The Y-tube olfactometer data presented here show that *C. storeyi* was attracted to VOCs from fresh, uninfested maize seedlings but was repelled by VOCs from *C. storeyi*-infested seedlings. Furthermore, VOCs from uninfested seedlings were more attractive than those from infested seedlings. Bioassays with synthetic compounds were limited to those induced by *C. storeyi* infestation, specifically those reported elsewhere as volatile semiochemicals involved in plant defense against other sucking insect pests, i.e., methyl salicylate, (*E*)-caryophyllene, (*E*)-β-farnesene, and TMTT. The identification of behaviorally active compounds involved in location of uninfested seedlings was not proceeded with, as this was outside the scope of the study. Methyl salicylate, (*E*)-caryophyllene, (*E*)-β-farnesene, and TMTT were significantly repellent when tested alone at a single dose (1 μg). Furthermore, addition of either methyl salicylate, (*E*)-β-farnesene, or TMTT at the same dose as an attractive blend of maize VOCs not only masked the attractiveness of the blend, but made it repellent to *C. storeyi*. Further dose-response studies are required to confirm the behavioral role of these compounds, particularly at much lower, ecologically relevant levels.

As stated above, although the results in this study represent one of the first reports of plant defense induction by leafhoppers, methyl salicylate, (*E*)-β-farnesene, and TMTT have all been reported elsewhere as volatile semiochemicals involved in plant defense against other sucking insect pests. A number of aphid species (Homoptera: Aphididae) have strong antennal electrophysiological responses to methyl salicylate, including *Rhopalosiphum padi*, *Sitobion avenae* and *Metopolophium dirhodum*, all of which have been shown to possess a specific olfactory neuron for this compound (Pettersson et al., 1994). Furthermore, methyl salicylate is a winter host plant component involved in mediating spring migration from winter to summer host plants (Pettersson et al., 1994). Induction of methyl salicylate production also has been reported from soybean, *Glycine max*, following exposure to the soybean aphid, *Aphis glycines*, with this compound being reported as an olfactory cue exploited by aphidophagous ladybirds (Zhu and Park, 2005). The sesquiterpene (*E*)-β-farnesene is an alarm pheromone for many aphid species (Pickett and Glinwood, 2007 and references therein). TMTT is a potent repellent for aphids, and an attractant for aphid and stinkbug parasitoids, when released by cotton, soybean and *Arabidopsis thaliana* following exposure to the plant activator *cis*-jasmone (Birkett and Bruce, unpublished data; Bruce et al., 2008; Moraes et al., 2009). Furthermore, aphid parasitoids possess a specific olfactory neuron for this compound (Bruce et al., 2008).

Methyl salicylate, (*E*)-β-farnesene, and TMTT have been shown to possess potent field activity against aphids. Application of slow-release formulations of methyl salicylate to wheat significantly reduced (by 30–40%) the overall number of aphids colonizing the crop (Pettersson et al., 1994), while application of controlled release dispensers of the same compound in hop yards and grape blocks resulted in enhancement of aphid biological control (James et al., 2005, James and Grasswitz, 2005; Zhu and Park, 2005). Application of slow-release formulations of *Hemizygia petiolata* essential oil, enriched in (*E*)-β-farnesene, reduces pea aphid populations on bean crops (Bruce et al., 2005). To our knowledge, there are no reports of using slow-release formulations of other insect-induced maize VOCs in the field. However, their constitutive release from intact plants, such as *Melinis minutiflora*, has been exploited in the development of the push-pull strategy for the control of lepidopterous stem borers in maize subsistence farms in East Africa (Kimani et al., 2000; Hassanali et al., 2008).

The behavioral activity of induced volatile semiochemicals against *C. storeyi* leafhoppers in this study suggests that they have potential for use in leafhopper control, and reduce the prevalence of plant diseases through reduced vector activity, either as outright repellents, or as attractants of natural enemies such as hymenopterous parasitoids (e.g. Moya-Raygoza et al., 2006). However, the release of such materials, either as slow-release formulations, or *via* genetically-transformed plants (Beale et al., 2006), is not feasible for resource-poor farmers based in Western Africa. Maize shows relatively high genetic and VOC variability, both intrinsically and in herbivore-induced emissions (Gouinguene et al., 2001; Degen et al., 2004). Work is currently underway to investigate the emission of the induced semiochemicals, i.e., methyl salicylate, (*E*)-β-farnesene, and TMTT, identified in this study, in local wild and elite, i.e., African cultivars, which could be developed for use in the manipulation of leafhopper or natural enemy behavior.

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Chemical Cues for Host Location by the Chestnut Gall Wasp, *Dryocosmus kuriphilus*

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Abstract *Dryocosmus kuriphilus* is one of the most damaging pests of *Castanea* spp. Behavioral, chemical, and electrophysiological investigations were employed to examine the role of plant volatiles for host location by this thelytokuos cynipid. Y-tube olfactometer bioassays showed that adult wasps are significantly attracted by *C. sativa* twigs with at least 1-hr-old mechanical damage. Odors of undamaged host seedlings, intact twigs, and twigs with a fresh mechanical damage were not attractive. Wasps were repelled by plant materials of the non-host *Prunus laurocerasus*. Fourteen compounds, mainly general green leaf volatiles, were identified in the head-space of attractive host plant twigs by gas chromatography coupled to mass spectrometry. All compounds elicited dose-dependent antennal responses in adult wasps. A synthetic blend comprising all identified compounds in the same ratio as in the attractive host source induced significant positive responses in Y-tube olfactometer bioassays. The study gives a basis for future identification of host plant attractants that could contribute to semiochemical-based monitoring and management practices of this pest.

Key Words Y-tube bioassay · Head-space analysis · SPME · GC-MS · Electroantennogram · Green leaf volatiles · Plant volatiles

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Introduction

The chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) is one of the most damaging pests of *Castanea* spp. (Abe et al., 2007). The species is univoltine and thelytokous (Zhu et al., 2007). Adults lay eggs in chestnut buds in early summer, and larvae remain latent until buds expand the following spring. Larvae induce the formation of leaf and twig galls that disrupt shoot elongation, reduce fruit yield by 50%–75%, and even cause the death of young trees as a result of severe consecutive attacks (Payne et al., 1983; Kato and Hijii, 1997). The effectiveness of chemical control is limited by the cryptic nature of the insect, which is in dormant buds for the majority of its life cycle, and by the difficulty of precisely detecting adult emergence. Moreover, chemical treatments often are impracticable due to the natural distribution of chestnut trees in steep terrain.

Gall-inducing cynipids are host-plant specific (Ronquist and Liljeblad, 2001). The oviposition of cynipids and subsequent gall development can be sensitive to host attributes including genotype, age, size, phenology, and nutritional status (Stone et al., 2002). The compositions of cynipid gall-wasp communities correlate with host plant chemistry (Abrahamson et al., 2003), and there is evidence that galls are manipulated by galling cynipids that enhance their food and protective value (Allison and Schultz, 2005).

Given the specificity of the host plant-cypnid interactions, plant volatiles are expected to play a crucial role in host recognition. Changes in host plant chemistry associated with galls provide cues for mate location by adult males in *Antistrophus rufus* Gillette (Hymenoptera: Cynipidae) (Tooker et al., 2002; Tooker and Hanks, 2004). Females of the same species respond positively to a four-component blend of plant monoterpenes in Y-tube olfactometer bio-

assays, thus suggesting that plant volatiles probably serve as an olfactory cue for host plant location (Tooker et al., 2005).

Identification of semiochemicals involved in host location by *D. kuriphilus* is of interest not only from an ecological perspective but also for development of novel pest control strategies. Host plant cues are particularly important in light of the thelytokous parthenogenesis characteristic of this species and the consequent lack of a sex pheromone system. Attractive semiochemicals could be incorporated into traps to detect adult wasps in new areas and to improve the timing of control measures.

In a preliminary study that tested the hypothesis that chestnut leaf volatiles are perceived by antennae of *D. kuriphilus* adults, electroantennographic (EAG) recordings showed that these insects can detect a wide range of plant volatiles. In addition, some EAG-active compounds were individually attractive to adult wasps in a Y-tube olfactometer bioassay (Germinara et al., 2009a). To better understand the chemical basis of host plant location by *D. kuriphilus* and in an attempt to find an attractive blend of host-related volatiles, comprehensive behavioral, chemical, and electrophysiological investigations were carried out.

Methods and Materials

Insects Chestnut galls containing immature stages of the chestnut gall wasp were collected in an infested orchard near Avellino (southern Italy) during June and July and kept in glass containers at 25±2°C, 55±5% relative humidity, and under natural light conditions. Newly emerged adults were collected daily and kept individually in glass vials (i.d. 2×8 cm) covered with a fine mesh net and starved for at least 12 hr prior to behavioral and electrophysiological tests.

Plants We used the host plant wild *Castanea sativa* Mill. and the non-host plant *Prunus laurocerasus* L. (Rosales). One meter long branches were cut from plants grown in a field near the University of Molise in Campobasso, Italy and placed individually into 2000-ml glass jars containing water. Branches were used for the experiments within 24 hr of cutting the branch from the tree. For some experiments, *C. sativa* seedlings about 1-yr-old (8–10 leaves, length 20–30 cm) were supplied by the Fruit Tree Unit of the Agricultural Research Council (Caserta, Italy). All plants used were in the flowering stage (June–July) when infestation by *D. kuriphilus* occurs in the field.

Chemicals (*E*)-2-hexenal, hexyl acetate, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, hexanol, (*E*)-3-hexenol, (*Z*)-3-hexenol, (*E*)-2-hexenol, (*Z*)-3-hexenyl butyrate, (*E*)-2-hexenyl butyrate, (*E*)-caryophyllene, (*Z*)-3-hexenyl hexanoate, methyl salicylate, geranyl acetate were purchased from Sigma-Aldrich (Milan, Italy), and were 97–99% pure. (*E*)-caryophyllene was an enantiomeric mixture.

Olfactometer Bioassays A glass Y-tube olfactometer (each arm 23 cm long at 75°C angle, stem 30 cm long, 3.0 cm i.d.) was used to examine the behavioral responses of *D. kuriphilus* adults to plant volatiles. Each arm of the Y-tube was connected to a glass cylinder (9 cm long and 3.0 cm i.d.) as an odor source container. The apparatus was put into an observation chamber (90×75×40 cm) and illuminated from above by two 36-W cool white fluorescent lamps providing uniform lighting (2500 lux) inside the tube. A purified (activated charcoal) and humidified airflow maintained at 60 ml/min by a flowmeter was pumped through each arm.

In order to determine an attractive source of host plant volatiles, four dual-choice experiments were performed with host plant materials: (1) host plant seedlings vs. clean air; (2) intact host plant twigs with two leaves and three buds vs. clean air; (3) host plant twigs 0–60 min after mechanical damage (fresh damage) vs. clean air; (4) host plant twigs 60–120 min after mechanical damage (old damage) vs. clean air.

To prevent contamination of volatiles emitted by host plant seedlings, the surface of the soil around the stem of the plant was covered with pieces of aluminum foil. Individual seedlings were equilibrated for 1 hr at room temperature in a two-part glass vessel 60 cm high and with a diameter of 32 cm. The upper part of the vessel was provided with a Drechsel-bottle head with the inlet connected to the flowmeter and the outlet to an arm of the olfactometer. An empty glass vessel was used as a control.

To test the specificity of the host plant signal, three additional tests were carried out: (1) intact non-host plant twigs vs. clean air; (2) non-host plant twigs with old mechanical damage vs. clean air; (3) host plant twigs with old mechanical damage vs. non-host plant twigs with old mechanical damage. Twigs used for the experiments were taken from the cut branches just before the start of the experiment, and their cut ends were placed in glass tubes containing water to maintain the physiological water content (Tasin et al., 2005). Each twig (ca. 2 g) included at least two leaves and three buds. Mechanical damage was achieved by cutting twigs into pieces (0.5 cm wide) (van Tol et al., 2002).

We determined the response of adult wasps to a synthetic blend comprising 14 identified compounds mixed in the same ratio as in the head-space sample of host plant twigs with old mechanical damage. In this case, the odor chamber contained a filter paper disk (0.5 cm^2) loaded with 5 µl of a 0.01, 0.1 or 1 M synthetic volatile blend dissolved in

mineral oil (Sigma-Aldrich, Milan, Italy), while the control chamber contained a filter paper disk loaded with 5 μl of mineral oil. Both disks were suspended in the center of the cross section.

Individual chestnut wasps were released at the open end of the stem. Each experiment lasted 10 min. A choice was recorded when the insect moved 3 cm up an arm of the Y-tube crossing the decision line (marked on both arms) and remained beyond that line for more then 30 sec. The time spent by test insects in each arm also was recorded.

After 5 individuals were tested, the olfactometer was cleaned with acetone and dried (200°C for 30 min). Treatments between arms were switched to avoid position bias. For each test stimulus at least 30 wasps were tested.

Sampling of Volatiles Volatiles emitted by *C. sativa* twigs were collected by Solid Phase Microextraction (SPME) using a manual SPME holder with a polydimethylsiloxane coated fiber (100 μm) (Supelco, Bellefonte, PA). A chestnut twig similar to those used for behavioral tests was cut into pieces (0.5 cm) on aluminum foil and was used immediately for the extraction, or was kept exposed to air at room temperature (23±1°C) for 60 min. Plant material then was enclosed in a 25 ml screw top mini flask with a PTFE thread Mininert™ Valve (Sigma-Aldrich, Milan, Italy) and a SPME was exposed to the head-space of the sample. Individual analyses were standardized following the method for volatile organic compounds described in Bech et al. (2008). The method parameters were P=permeation time=1 min, E= exposure time=60 min, S=storage time=30 sec, and T= thermal desorption of SPME fiber time=15 min. Before each extraction, the fiber was conditioned for 30 min in a GC injection port at 250°C. For both fresh and old mechanical damage, samples from five different plants were analyzed.

Coupled Gas Chromatography-Mass Spectrometry (GC-MS) SPME extracts were analyzed in a Fisons 8000 series gas chromatograph coupled with a Fisons MD 800 quadrupole mass spectrometer using a CPWAX 52 CB capillary column (30 m×0.32 mm i.d., 0.25 μm film thickness) (Ruther, 2000). The oven temperature was kept at 40°C for 3 min, programmed to rise 3°C min^{-1} to 230°C and hold at 230°C for 30 min. Injection temperature was kept at 240°C. Helium was used as carrier gas at a flow rate of 2 ml min^{-1}. Mass spectra were recorded in the electron impact mode (EI) at 70 eV and at a source temperature of 200°C. Mass range was from 25 to 450 m/z at a scan time of 0.9 sec and an interscan delay of 0.1 sec. Compounds were identified tentatively by comparison of mass spectra with those of authentic samples in the NIST database (2005) and confirmed by comparison of mass spectra and retention times with those of authentic reference compounds. In order to determine the relative composition of plant extracts, for each analysis the sum of peak areas was set to 100% and the mean contribution of each individual compound was calculated.

Electroantennography (EAG) Electrophysiological activity of identified compounds to adult wasps was determined by EAG (Germinara et al., 2009a). The head of a 1-d-old specimen was cut off and a glass pipette filled with 0.1 M KCl solution, which served as the neutral electrode, was inserted into its base. The distal end of the last antennal segment was put in contact with the end of a similar pipette (0.2–0.3 mm i.d.) serving as the recording electrode. AgCl-coated silver wires were used to maintain the electrical continuity between the antennal preparation and an AC/DC UN-6 amplifier in DC mode connected to a PC equipped with the EAG 2.0 program (Syntech Laboratories, Hilversum, The Netherlands). A stream of charcoal-filtered humidified air (500 ml/min) was directed constantly onto the antenna through a stainless steel delivery tube (1 cm i. d.) with the outlet positioned at approximately 1 cm from the antenna.

Just before the experiment, solutions of authentic standards of identified compounds were applied to filter paper (Whatman No. 1) strips (1 cm^2) inserted in a Pasteur pipette (15 cm long) and used as an odor cartridge. For each compound, seven doses ranging from 10^{-3} to 40 μM in 10 μl mineral oil were tested. Over 1 sec, 2.5 cm^3 of vapor from an odor cartridge were blown by a disposable syringe into the air stream flowing over the antennal preparation. Stimuli were applied in ascending doses. Control (10 μl of mineral oil) and standard stimuli (10 μl of 1 M *Z*3-hexen-1-ol solution) were applied at the beginning of the experiment, and after each group of 3 randomly selected odors. Intervals between stimuli were 30 sec. For each compound, EAG responses were recorded from 5 antennae of different insects.

Data Analysis The amplitude (mV) of the EAG response to each test stimulus was adjusted to compensate for solvent and/or mechanosensory artefacts by subtracting the mean EAG response of the two nearest mineral oil controls (Raguso and Light, 1998). To compensate for the decrease of the antennal responsiveness during the experiment, the resulting EAG amplitude was corrected according to the EAG response to the standard stimulus (Den Otter et al., 1991).

In dose-response curves, the activation threshold was considered to be the lowest dose at which the lower limit of the standard error of the mean response was greater than the upper limit of the standard error for the lowest dilution tested (Sant'ana and Dickens, 1998). Saturation level was taken as the lowest dose at which the mean response was equal to or less than the previous dose (Germinara et al., 2009b).

Table 1 Response of *Dryocosmus kuriphilus* adults in a Y-tube olfactometer to different odor sources of host (*Castanea sativa*) and non-host (*Prunus laurocerasus*) plants

Odor sources	N^a	First choice			Number of minutes spent in arm (mean ± SE)			
		Treated[b]	χ^2	*P*-value	Treated	Control	*t*-value	*P*-value
Host seedling vs. control air	32 (25)	0.56	0.36	0.549	2.3±0.4	2.8±0.6	−0.615	0.544
Host intact twigs vs. control air	33 (25)	0.56	0.36	0.317	2.5±0.4	2.2±0.4	0.515	0.611
Host twigs with fresh damage vs. control air	34 (30)	0.60	1.20	0.273	2.9±0.4	2.7±0.5	0.207	0.837
Host twigs with old damage vs. control air	33 (30)	0.80	10.80	0.001	5.1±0.6	2.4±0.6	2.688	0.012
Non-host intact twigs vs. control air	30 (24)	0.33	2.67	0.102	2.6±0.2	3.6±0.2	−2.108	0.046
Non-host twigs with old damage vs. control air	30 (23)	0.17	9.78	0.002	1.0±0.2	6.6±0.5	−8.338	<0.001
Host twigs with old damage vs. non-host twigs with old damage	34 (27)	0.85	13.37	<0.001	3.9±0.5	1.0±0.3	5.129	<0.001

[a]Total sample size (*N*, number of individuals that made a choice in parentheses)

[b]Proportion of individuals (of those that made a choice) that chose the treated arm first

A χ^2 test was employed to determine the significance of differences between the number of wasps choosing the treatment or control arm of the olfactometer. A paired-sample *t*-test was used to analyze the differences between the time spent by wasps in each arm.

Results

Behavioral Response to Plant Materials In behavioral tests with plant materials, the percentage of adult wasps responding was generally high. Odors from undamaged host seedlings, intact twigs, and twigs with fresh mechanical damage did not elicit a significant response from adult wasps measured either as first choice or as time spent in the treatment arm when clean air was the alternative (Table 1). Insects presented with host twigs with old mechanical damage vs. clean-air control exhibited a significant preference for the damaged twigs (χ^2=10.80, *d.f.*=1, *P*=0.001) and spent significantly more time in the treatment arm (Table 1).

When insects could choose between non-host intact twigs and clean air, there was no preference but they spent significantly more time in the control arm (Table 1). Moreover, insects presented with non-host plant twigs with old mechanical damage vs. clean air control significantly preferred and spent more time in the control arm (Table 1). A significant positive response to host twigs with old mechanical damage was confirmed also in the presence of non-host twigs with a similar damage (Table 1).

Chemical Analyses GC-MS analysis revealed 15 volatile compounds in the SPME extracts from damaged chestnut twigs (Table 2). Fourteen of them were confirmed by comparison of retention times and mass spectra to those of authentic samples, while one compound remained unidentified. Chemical analysis showed differences between the volatiles collected from chestnut twigs with fresh or old mechanical damage. The volatile blend collected from freshly damaged twigs lacked the green leaf aldehyde (*E*)-2-hexenal. One hour after mechanical damage, there was a significant reduction in the relative amount of (*Z*)-3-hexenyl acetate and a significant increase in that of hexanol and (*E*)-2-hexenol (Table 2).

Table 2 Mean relative peak areas (±SE) of compounds detected in the head-space of damaged chestnut twigs. Volatiles were collected by SPME for 60 min immediately (fresh) or 1 hr (old) after mechanical damage and subsequently analyzed by gas chromatography coupled with mass spectrometry. Asterisks indicate significant differences between fresh and old damage (*t*-test, * $P<0.05$; ** $P<0.01$)

Compounds	Fresh damage [a]	Old damage
(*E*)-2-hexenal	0.0±0.0	1.9±0.9 *
Hexyl acetate	5.7±0.2	5.4±0.7
(*Z*)-3-hexenyl acetate	70.9±2.9	51.9±4.1**
(*E*)-2-hexenyl acetate	2.5±0.6	4.5±0.7
Hexanol	0.8±0.1	2.8±0.7*
(*E*)-3-hexenol	0.1±0.1	0.2±0.1
(*Z*)-3-hexenol	12.5±1.8	18.3±1.8
(*E*)-2-hexenol	0.3±0.1	1.5±0.4 *
(*Z*)-3-hexenyl butyrate	2.5±0.4	5.3±3.1
(*E*)-2-hexenyl butyrate	0.1±0.1	0.7±0.4
(*E*)-Caryophyllene	1.4±0.8	0.3±0.2
(*Z*)-3-hexenyl hexanoate	0.8±0.2	2.4±0.8
- [b]	1.1±0.2	1.2±0.2
Methyl salicylate	0.7±0.4	0.6±0.3
Geranyl acetate	0.6±0.2	2.8±1.3

[a]For each extraction, percentages are calculated by dividing the peak area of a compound by the total area of compounds detected

[b]Unidentified

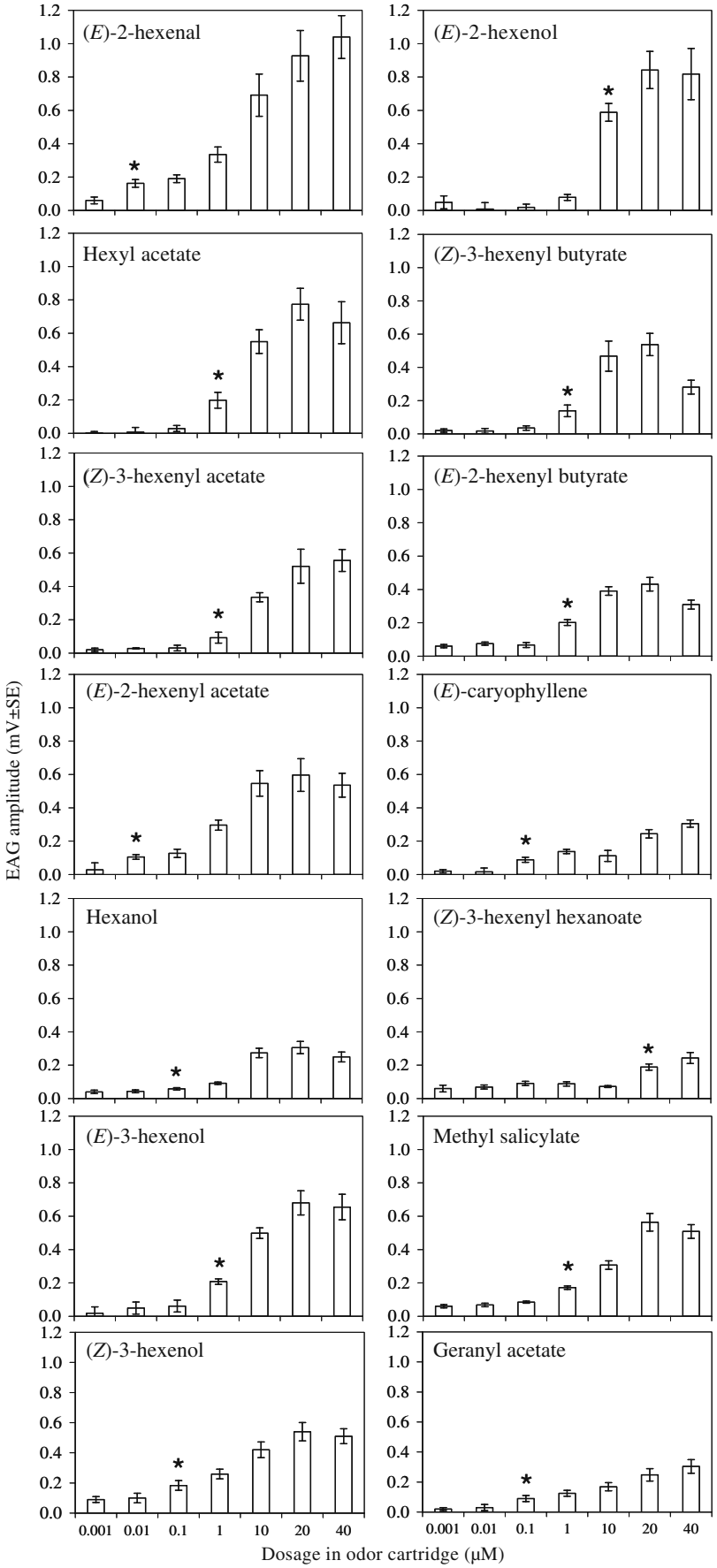


Fig. 1 Electroantennogram dose-response profiles of *Dryocosmus kuriphilus* adults (n=5) to compounds identified in the head-space of damaged *Castanea sativa* twigs. Asterisks indicate the activation thresholds

EAG Dose-Response Characterization The sensitivity of adult *D. kuriphilus* antennae toward increasing concentrations of authentic standards of identified compounds are reported in Fig. 1. For all compounds, the amplitude of the EAG response increased with dose. The lowest activation thresholds were recorded for (*E*)-2-hexenal and (*E*)-2-hexenyl acetate at the 0.01 μM dose. The activation threshold for the remaining compounds was 0.1 μM for hexanol, (*Z*)-3-hexenol, (*E*)-caryophyllene, and geranyl acetate, 1 μM for hexyl acetate, (*Z*)-3-hexenyl acetate, (*E*)-3-hexenol, (*Z*)-3-hexenyl butyrate, (*E*)-2-hexenyl butyrate, and methyl salicylate, and 10 and 20 μM for (*E*)-2-hexenol and (*Z*)-3-hexenyl hexanoate, respectively.

The amplitude of the EAG response to hexyl acetate, (*E*)-2-hexenyl acetate, hexanol, (*E*)-3-hexenol, (*Z*)-3-hexenol, (*E*)-2-hexenol, (*Z*)-3-hexenyl butyrate, (*E*)-2-hexenyl butyrate, and methyl salicylate decreased from 20 to 40 μM doses indicating saturation of receptors at the lowest dose.

Behavioral Response to a Synthetic Blend The behavioral responses of adult wasps to different doses of a multicomponent blend comprising the 14 commercially available authentic standards in the same ratio as in the head-space sample of host plant twigs with old mechanical damage are reported in Table 3.

At the 0.05 and 0.5 μM doses, a significant attraction of adult wasps, either as first choice or time spent in the treatment arm of the olfactometer was recorded. At the 5 μM dose, adult wasps did not show any significant preference for either the treatment or the control arm.

Discussion

In Y-tube olfactometer bioassays, adults of the chestnut gall wasp were attracted by odors of leaf- and bud-bearing *C. sativa* twigs with at least one-h-old mechanical damage. Odors of undamaged host seedlings, intact twigs, and twigs with fresh mechanical damage were unattractive. Moreover, insects were able to discriminate between host and non-host plant materials, suggesting specificity of host-plant chemical signals. Chemical analysis showed that the blend of volatiles emitted by artificially damaged *C. sativa* twigs changed significantly over time. From the first to the second hour after mechanical damage, the additional presence of (*E*)-2-hexenal was detected. In addition, there was a significant decrease in the relative proportion of (*Z*)-3-hexenyl acetate and a significant increase in that of hexanol and of (*E*)-2-hexenol. These changes might account for the attractiveness of old mechanically damaged twigs compared to the neutral effect of the freshly damaged ones.

The majority of compounds identified in the head-space of attractive host-plant samples were C_6 alcohols, esters, and the aldehyde (*E*)-2-hexenal. These so-called green leaf volatiles (GLVs) contribute to the "green odor" of the leaves of numerous plant species. GLVs may be produced constitutively or induced by leaf damage caused abiotically or by herbivory (Visser, 1986; Loughrin et al., 1996; Turlings et al., 1998; Ruther et al., 2002) and by light-dark transitions (Graus et al., 2004; Chamberlain et al., 2006). Additional components were the terpenoids (*E*)-caryophyllene and geranyl acetate and the aromatic methyl salicylate. These compounds are released in response to plant damage (Takabayashi et al., 1994; Bolter et al., 1997; Hoballah and Turlings, 2005).

Volatile compounds emitted upon mechanical and/or herbivore damage are important in the host selection process for several phytophagous insects (Loughrin et al., 1996; Dickens, 2000; Kalberer et al., 2001; van Tol et al., 2002). In some cases, a neutral effect of undamaged host plant material also has been recorded (Bolter et al., 1997; Ruther et al., 2000; van Tol et al., 2004). The advantages of adult *D. kuriphilus* being attracted to a damaged plant remain unclear.

All identified compounds elicited EAG dose-dependent responses with different activation thresholds. At doses from 0.05 to 0.5 μM, a synthetic blend comprised of all 14 EAG-active volatiles in the same ratio as in the head-space

Table 3 Response of *Dryocosmus kuriphilus* adults in a Y-tube olfactometer to increasing doses of a synthetic blend comprising all identified compounds in the same ratio as in the head-space sample of host plant twigs with old mechanical damage

Dose (μM)	*N*[a]	First choice			Number of minutes spent in arm (mean ± SE)			
		Treated[b]	χ^2	*P*-value	Treated	Control	*t*-value	*P*-value
0.05	33 (25)	0.72	4.84	0.028	3.3±0.3	2.2±0.3	2.302	0.030
0.5	35 (25)	0.80	9.00	0.003	3.3±0.5	1.4±0.3	2.535	0.018
5	36 (25)	0.52	0.04	0.841	2.2±0.4	2.5±0.5	−0.286	0.777

[a]Total sample size (*N*, number of individuals that made a choice in parentheses)

[b]Proportion of individuals (of those that made a choice) that chose the treated arm first

of the attractive host plant materials elicited a significant attraction in adult wasps, suggesting that these compounds are involved in locating host plants. One compound could not be identified and therefore it was not included in the EAG and behavioral tests. However, it seems unlikely that this compound may play a role in host plant location since its relative proportion in the natural blend did not change significantly over time.

In a previous study, (*E*)-2-hexenal did not elicit a positive response in the adult wasps (Germinara et al., 2009a), therefore, the additional presence of this compound in the odor blend of old mechanically damaged twigs could not explain its attractiveness as compared to the blend of freshly damaged twigs. This, along with the ubiquitous nature of all identified chestnut volatiles, suggests that host location by the chestnut wasp is more likely mediated by a host-specific ratio of ubiquitous volatiles rather than by a single compound. The exploitation of ubiquitous compounds even by the monophagous *D. kuriphilus* supports the hypothesis of ratio-specific odor recognition as the most prevalent mechanism mediating host plant location by phytophagous insects (Visser, 1986; Bruce et al., 2005).

Adult wasps were significantly repelled by non-host volatiles, indicating that these compounds may act as antagonists. This evidence is in accordance with previous reports on the tendency of specialists to be deterred (Bernays et al., 2000) or repelled (Linn et al., 2005) by non-host compounds. The capability of the adult wasps to actively avoid non-host plant volatiles and not just to passively ignore them could be important for optimizing host location, and it could have important evolutionary consequences (Forbes et al., 2005; Feder and Forbes, 2007). Moreover, repellent compounds from non-host plants may have potential for the use in the control of insect pests (Ukeh et al., 2010).

Many previous studies have shown that only a small number of host plant volatiles out of all of those electrophysiologically active to an insect are involved in host location (Rojas, 1999; Dickens, 2000; Fraser et al., 2004; Tasin et al., 2006), suggesting that not all 14 components of the synthetic blend are necessary for attraction. Laboratory and field studies examining the behavioral responses of *D. kuriphilus* with various dosages of individual synthetic compounds and their mixtures are in progress to find out those that are crucial in host plant location. Identification of such compounds may contribute to the development of effective monitoring and management tools for this pest.

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Qualitative Variation in Proanthocyanidin Composition of *Populus* Species and Hybrids: Genetics is the Key

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Abstract The literature on proanthocyanidins (tannins) in ecological systems is dominated by quantitative studies. Despite evidence that the qualitative characteristics (subunit type, polymer chain length) of these complex polyphenolics are important determinants of biological activity, little is known about genetic and environmental controls on the type of proanthocyanidins produced by plants. We tested the hypothesis that genetics, season, developmental stage, and environment determine proanthocyanidin qualitative characteristics by using four *Populus* "cross types" (narrowleaf [*P. angustifolia*], Fremont [*P. fremontii*], F1 hybrids, and backcrosses to narrowleaf). We used thiolysis and HPLC analysis to characterize the proanthocyanidins, and found that genetics strongly control composition. The narrowleaf plants accumulate mixed procyanidin/prodelphinidins with average composition epicatechin$_{11}$-epigallocatechin$_8$-catechin$_2$-catechin$_{(terminal)}$. Backcross genotypes produce mixed procyanidin/prodelphinidins similar to narrowleaf, while Fremont makes procyanidin dimers, and the F1 plants contain procyanidin heptamers. Less striking effects were noted for genotype × environment, while season and developmental zone had little effect on proanthocyanidin composition or chain length. We discuss the metabolic and ecological consequences of differences in condensed tannin qualitative traits.

Key Words *Populus* · Condensed tannin · Genetic variation · Polyphenolic · Proanthocyanidin · Tannin composition · Thiolysis

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Introduction

Secondary plant products including phenolics have important roles in regulating the interactions between plants and other organisms, in determining the responses of plants to abiotic stresses, and in governing ecosystem processes. As a consequence, investigation of how genetics, development, and environment control production of secondary compounds is essential for understanding plant physiology and ecology. Ideally, studies of secondary product accumulation include both qualitative and quantitative measures. Qualitative assessments that reveal production of specific compounds are important (Yarnes et al., 2008), since bioactivity is usually associated with specific compounds, not classes of compounds. Quantitative assays are useful for establishing metabolic flux and utilization of resources such as C and N to make broad classes of compounds (Arnold and Schultz, 2002).

Studies of secondary compound accumulation often target phenolics because of their wide distribution and diverse functions. In general, phenolic compounds can be divided into the low molecular weight phenolics and the polymeric polyphenolics (tannins). The low molecular weight phenolics include numerous compounds such as phenolic acids, glycosides, esters, and flavonoids, while the

polyphenolics generally are classified as either hydrolyzable tannins (gallotannins and ellagitannins) or proanthocyanidins (condensed tannins) (Yoshida et al., 2005).

Genetics, development, environment, and their interactions strongly influence production of low molecular weight, nontannin phenolics. Levels of phenolic glycosides in *Populus* are influenced by genotype and by developmental and environmental factors such as age (Donaldson et al., 2006), CO_2 and nutrient levels (Lindroth et al., 2001), and defoliation or leaf damage (Stevens and Lindroth, 2005). Qualitative profiles of low molecular weight phenolics also are controlled by the interaction of genetics with developmental and environmental cues. Genetic control predominates over phenolic glycoside composition of parent and F1 offspring of *Salix*, with inheritance under directional dominance (Orians et al., 2000). In contrast, in three species of *Betula*, juvenile leaves had similar profiles of low molecular weight phenolics, but mature tissue had species-specific compositions, suggesting ontogenetic influence on genetically specified phenolic composition (Laitinen et al., 2005).

Regulation of hydrolyzable tannin accumulation is similar to regulation for simple phenolics. Some oaks accumulate high levels of hydrolyzable tannin (bur, pin, or willow) while other species (cherrybark, Northern red) accumulate far smaller amounts (Foss and Rieske, 2003), suggesting an overall genetic constraint on the total quantity of hydrolyzable tannin that can be produced. Within that limit, both simple phenolics and complex ellagitannins are accumulated in patterns that are genetically controlled (Yarnes et al., 2006), with distinct tannin chemotypes identified in a population of *Quercus gambelii x Q. grisea* (Yarnes et al., 2008). During the growing season, oak leaves produce compounds sequentially in a pattern reflecting proposed biosynthetic relationships, with simple gallotannins appearing earlier in the growing season and ellagitannins accumulating later, thus demonstrating ontogenetic control over qualitative composition of hydrolyzable tannins (Salminen et al., 2004). Levels of accumulated hydrolyzable tannins are influenced by environmental conditions such as light and CO_2, which independently modulate the amounts of gallotannins and ellagitannins produced in *Acer saccharum* (Agrell et al., 2000).

In contrast to simple phenolics and hydrolyzable tannins, our understanding of the regulation of proanthocyanidin production is based almost entirely on quantitative assessments. Many studies have demonstrated that overall proanthocyanidin level is genetically controlled, and that environmental conditions may modify those levels. For example, in *Populus* (Osier and Lindroth, 2006), *Salix* (Orians et al., 2000), and *Betula* (Laitinen et al., 2000), the quantity of proanthocyanidin accumulated is genetically determined. Levels of proanthocyanidins in *Populus*, *Salix*, *Betula*, and *Fagus* are affected by environmental conditions such as levels of light, nutrients, CO_2, and ozone (Lindroth et al., 2001; Lower and Orians, 2003; Karonen et al., 2006; Osier and Lindroth, 2006). Almost nothing is known about what factors dictate accumulation of specific proanthocyanidins. Subunit composition, interflavan bond position, and chain length are important variables in determining proanthocyanidin activity (Ayres et al., 1997; Kraus et al., 2003), but little is known about how these features are determined. Koupai-Abyazani et al. (Koupai-Abyazani et al., 1993a) reported that in *Sainfoin*, subunit composition of proanthocyanidins changed as the leaves matured, suggesting developmental influence over proanthocyanidin accumulation. However, technical difficulties in differentiating individual proanthocyanidins have prevented us from establishing whether qualitative differences in proanthocyanidins are controlled genetically, developmentally, or environmentally. This limitation has slowed our understanding of the specific functions that proanthocyanidins contribute to biological systems.

We have conducted detailed qualitative studies of proanthocyanidin accumulation in *Populus*, examining the effects of genetics, season, developmental stage, and environmental factors. Wide-ranging "genes-to-ecosystems" studies of the *Populus* model system have provided insight into the relationships between plant genetics, chemistry and ecology (Whitham et al., 2006). *Populus* is an attractive taxon for studies of proanthocyanidin accumulation because its species do not produce hydrolyzable tannins. *Populus* does accumulate small phenolic glycosides, but via metabolic pathways distinct from the flavonoid metabolism that yields proanthocyanidins (Tsai et al., 2006). Recently Rehill et al. (2006) used quantitative assays to examine proanthocyanidin accumulation in four *Populus* "crosstypes" (two species and two hybrid types), across season, developmental stage, and location of garden plot. Similar to small phenolics, the quanitity of proanthocyanidins accumulated in leaf tissue was strongly genetically determined. The levels of proanthocyanidins in the F1 and backcross hybrids generally were intermediate to the parents (narrowleaf [*P. angustifolia*] and Fremont [*P. fremontii]*), suggesting additive inheritance. As noted for other phenolics, environmental factors including season and location modulated the levels of proanthocyanidins. In addition, significant variation in quantity of proanthocyanidin was associated with foliar developmental zone (i.e., mature and juvenile zones on individual trees) indicating developmental control of proanthocyanidin biosynthesis. Rehill et al. (2006) did not examine qualitative features of the *Populus* proanthocyanidins.

We hypothesized that qualitative characteristics of the proanthocyanidins in *Populus* would be controlled mainly

by genetics, just as composition of low molecular weight phenolics is mainly under genetic control. We predicted that subunit composition and chain length in F1 and backcross *Populus* would reflect the proanthocyanidins of their parents. We predicted that season, developmental stage, and environmental factors would have minimal effects on *Populus* proanthocyanidin composition. We tested these hypotheses by using leaf samples from four crosstypes of *Populus* collected across a growing season, at two developmental stages, and from gardens in three locations. Proanthocyanidins were extracted from the tissue, chemically derivatized with phenylmethanethiol (toluene-α-thiol), and analyzed by high performance liquid chromatography to establish subunit composition and average chain length.

Methods and Materials

Plants We sampled leaf tissue from the four crosstypes of *Populus* described in Rehill et al. (2006): Fremont (*P. fremontii*), narrowleaf (*P. angustifolia*), F1 hybrids, and the backcrosses to narrowleaf. Genetic introgression in this system is unidirectional: hence the F1 hybrids cross only with narrowleaf, not with Fremont. All plant tissue was collected and processed as described in Rehill et al. (2006). Briefly, leaves were clipped from their petioles, flash-frozen between blocks of dry ice, and shipped to the University of Wisconsin, Madison, Wisconsin (U.S.A.). The frozen leaves were freeze-dried, then ground in a Wiley mill (#40 mesh), and stored at −20°C. Samples were sent to Miami University, Oxford, Ohio (U.S.A.) for qualitative analysis via thiolysis.

To evaluate the effects of season on the composition of proanthocyanidins, leaf samples from mature branches of 7–10 trees from each of the four cross types were collected from the common garden at the Ogden Nature Center (ONC) in late May, late June, and late July 2003 (105 samples). The ONC common garden (41°14′48″ N, 111°59′59″ W; elevation 1300 m) represents *Populus* genotypes sampled from across a 13-km hybrid zone and from pure zones along the Weber River. Replicated genotypes of each of the four *Populus* cross types were planted in a random arrangement in the garden.

To evaluate the role of developmental stage on composition of proanthocyanidins, in June 2007 leaves were collected from both the juvenile zone and the mature zone of four trees from each of five narrowleaf genotypes (1000, 1008, 1020, He-10, WC5) for a total of 40 samples. The developmental stage of the leaves was determined by the presence of catkins or catkin scars on the branches (Rehill et al., 2006). Leaves used for comparisons among developmental stages were collected only from trees growing in the ONC garden.

To evaluate the effects of environmental conditions on composition of proanthocyanidin, leaves were collected from mature branches of the same genotypes planted at three different common garden locations: North Pit, ONC, and Taggart gardens, all planted between 1981 and 1992. From all three common gardens, leaves from 2 to 5 plants from each of the following genotypes were collected in June 2007: backcross genotype 10, narrowleaf genotypes 1000, 1008, and 1019 (41 samples). The ONC garden, described above, is dry most of the year, but a small creek adjacent to the garden provides water intermittently. The North Pit garden (41°08′00″ N, 111°54′05″ W; elevation 1395 m) is at the entrance to Weber Canyon on a gravelly slope, near the edge of a gravel pit at the mouth of Weber Canyon. The pit garden is dry, and is exposed to constant, strong winds coming out of Weber Canyon. Neither ONC nor North Pit has supplemental irrigation. The Taggart common garden (41°03′ 38″ N, 111°34′25″ W; elevation 1587 m) is located 6 km east of Morgan, UT, USA in the channel of the Weber River. Therefore, temperatures are cooler and water is constantly available at Taggart, while both the North Pit garden and the Ogden Nature Center garden have similar, warmer temperatures and little water.

Proanthocyanidin Extraction For samples from narrowleaf, F1, and backcross samples, about 10 mg of freeze-dried, ground tissue were weighed into a 1.7 ml microfuge tube. The samples were mixed with 250 μl of chloroform:methanol (2:1, v/v) with constant stirring for 30 min to remove chlorophyll and pigments, centrifuged for two min at 9300 × *g*, and the supernatants were discarded. The remaining tissue was treated with 250 μl ethyl acetate with constant stirring for 15 min to remove the remaining pigments and free phenolics. Samples were centrifuged as above, and ethyl acetate was discarded. After a second ethyl acetate extraction, tissues were briefly dried under flowing nitrogen gas to remove any remaining ethyl acetate. Tissues were then extracted with 200 μl of methanol with constant stirring for 60 min.

The extraction protocol was modified for Fremont samples to accommodate the very low level of proanthocyanidins in this cross type. Approximately 40 mg of tissue were weighed into 1.7 ml microfuge tubes and extracted with twice the volume of solvents compared with the other cross types (500 μl chloroform methanol, 500 μl ethyl acetate, 400 μl methanol). The remaining steps in the extraction were as described above.

Thiolysis Each plant extract was reacted with phenylmethanethiol (toluene-α-thiol) to yield terminal flavan-3-ols and the thiol derivatives of the extender flavan-3-ols (Guyot et al., 2001a). The reaction was started by mixing 100 μl of the plant extract with 10 μl of hydrochloric acid in methanol (32% by volume) and 24 μl of phenyl-

methanethiol in methanol (5% by volume). The reaction mixtures were incubated at 40°C for 30 min, and reactions were stopped by placing samples at −5°C for 5 min. Samples were then stored at −80°C until analysis, with all analyses conducted within 24 h of completing the reactions.

In order to determine proanthocyanidin composition, we needed standards for the flavan-3-ol thiol derivatives. We prepared the standards by carrying out thiolysis (Guyot et al., 2001b) on the proanthocyanidins from well-characterized plant sources, isolating the thiol products by column chromatography (Hagerman and Butler, 1980) and establishing identity of each product by mass spectrometry (Souquet et al., 1996). We then used these standards to identify thiol adducts in our *Populus* extracts by retention time on HPLC. Epicatechin thiol was obtained from *Sorghum bicolor* grain (Gupta and Haslam, 1978), catechin thiol from catechin trimer C-2 (Kolodzieg, 1990), epigallocatechin thiol from sainfoin leaves (Koupai-Abyazani et al., 1993b), and gallocatechin thiol from *Populus tremuloides* leaves (Hagerman, unpublished).

The thiolysis reaction products for standards and experimental *Populus* samples were run on reversed phase HPLC on an Agilent 1100 system with ChemStation Rev. A.09.03 software (Agilent, Santa Clara, CA, USA). The column was an Agilent Zorbax RP-8 column, 4.6×150 mm with 5 μm packing. The chromatography employed gradient programming with a gradient comprised of 0.13% trifluoroacetic acid (TFA) in water (v/v) and 0.1% TFA in acetonitrile (v/v) as the mobile phase at a flow rate 0.5 ml/min. For the first 3 min of each run, the mobile phase was maintained with 15% of the organic phase. Linear gradients increased solvent strength to 20% organic phase at 8 min, and to 30% at 10 min. Between 28 and 32 min, a linear gradient brought the mobile phase to 70% organic. A linear gradient from 37 to 40 min returned the mobile phase to the initial condition of 15% organic, and the column was re-equilibrated from 40 to 45 min. The same solvents with a slightly different gradient program were used in a few preliminary analyses employing a Zorbax C-18 column.

Two modes of detection were used: UV at 220 nm and electrochemical detection. The 12 channel CoulArray detector was employed over the voltage range from 180 to 840 mV with 60 mV increments between channels (ESA Chelmsford, MA, USA). The chromatograms were integrated automatically by using the ChemStation Rev.A.09.03 software for the UV data and Coularray for Windows Data Processing ver. 2.00 (ESA Chelmsford MA, USA) software for the electrochemical data. Baselines were set to accommodate changes in background absorbance with the gradient.

To confirm the identity of the standards, HPLC-MS was run under the same conditions but substituting acetic acid for TFA in the mobile phase. The Agilent HPLC was interfaced with an Esquire LC ion trap instrument (Bruker Daltonics, Billerica, MA USA). The electrospray ionization source (ESI) was operated in negative mode. A nominal target mass was set to 1000 before fine tuning. The capillary, skimmer 1, and trap drive voltages were −100, −30, and 45 V, respectively. The ion charge control was on with a target of 30,000. The 300°C nitrogen dry gas flow rate was 4 L/min and the nebulization gas pressure was 11 psi. Each data point in the spectrum consisted of an average of 4 scans over a mass range of 120–1000 m/z.

Acid Butanol Analysis To evaluate soluble and cell wall bound phenolics in the four cross types of *Populus*, 20 mg samples of tissue were extracted 3 times with 5 ml of aqueous acetone (3:7, water: acetone, v/v) in 50 ml falcon tubes. Subsamples (25 or 50 μl) of each extract (containing soluble proanthocyanidins) were reacted with 800 μl acid butanol (5% concentrated HCl in butanol, v/v) and 33 μl of iron reagent (2% ferric ammonium sulfate in 2 N aqueous HCl) for 10 min in a boiling water bath (Porter et al., 1986). The cell wall debris (containing bound proanthocyanidins), remaining after the exhaustive extraction, was reacted with 45 ml acid butanol and 1.8 ml ferric ammonium sulfate for 10 min in a boiling water bath with continuous stirring of the suspension. After heating, samples were cooled, and absorbances were determined at 550 nm.

Statistics The experimental designs employed were orthogonal. Mixed model analysis of variance (ANOVA) models with least squares estimation were used to analyze compositional data for proanthocyanidins, via PROC MIXED (SAS version 9.1 for Windows). Effects were tested for three extenders (epicatechin, epigallocatechin, and catechin) and for chain length. Gallocatechin extender data were not statistically analyzed because this trace component was found only in relatively few backcross and narrowleaf samples from June 2003. Individual trees served as replicates for assessment of proanthocyanidins among genotypes, developmental zones, and genotype × zone interaction effects. Genotype was nested within garden with individual trees serving as replicates for the assessment of proanthocyanidins across genotypes and gardens (G × E). For comparisons of proanthocyanidins across months within and between crosstypes, we used repeated measures ANOVA with month and crosstype as fixed effects and location as the random effect.

Results

Validation of Analytical Methods Thiolysis is a well-accepted method for establishing composition and chain

length of proanthocyanidins. The acid-catalyzed oxidative cleavage of 4,6 or 4,8 interflavan bonds yields a carbocation intermediate that reacts with the nucleophilic phenylmethanethiol to yield a thiol-derivitized flavan-3-ol. Under optimized conditions (Guyot et al., 2001a), the polymer is degraded completely to thiol adducts for all extender groups. The terminal unit is released as the underivitized flavan-3-ol.

Commercially available flavan-3-ols (Fig. 1) were used to determine retention times for the epimeric pairs of the possible terminal units (Table 1). The order of elution was the same for either the C18 or C8 column, and was consistent with the trihydroxylated (delphinidin-type) B ring (gallocatechin or epigallocatechin) increasing the polarity of the compounds relative to the dihydroxylated (cyanidin-type) B ring (catechin or epicatechin).

We prepared the thiol adducts of the four common flavan-3-ols (catechin thiol, epicatechin thiol, gallocatechin thiol, and epigallocatechin thiol (Fig. 1)) from plant tannins of known composition, and confirmed the identity of each by negative ion mode electrospray MS (Table 1). On the C-8 column the retention times of the thiols were inverted within the epimeric pairs compared to the free flavan-3-ols, so that although catechin (2R, 3S) eluted earlier than epicatechin (2R, 3R), catechin thiol eluted later than epicatechin thiol (Table 1). In contrast, using a C18 column we found that the epimeric pairs of the flavan-3-ols and their corresponding thiol adducts eluted in the same order (Table 1). With either type of column, the prodelphinidin-type thiols (gallocatechin, epigallocatechin) eluted earlier than the procyanidin-type compounds (catechin, epicatechin), similar to the order for the simple flavan-3-ols. It clearly is important to identify carefully each member of the epimeric pair for any HPLC system since small changes in chromatographic conditions can change the relative retention times.

We quantitated the flavan-3-ol terminal units and the thiol extender units based on detection at 220 nm and peak integration. We ran standard curves for the commercial flavan-3-ols and for the isolated thiols, establishing that the UV-response is equivalent on a molar basis for these four flavan-3-ols and their thiol adducts. For plant tissues that contained very low levels of proanthocyanidins, the UV peaks were too small to integrate accurately. For these samples, we used electrochemical detection to achieve a four-fold increase in sensitivity. Using a coularray detector, each compound was detected in a voltage window that gave the best linear response. The voltage windows were 240–

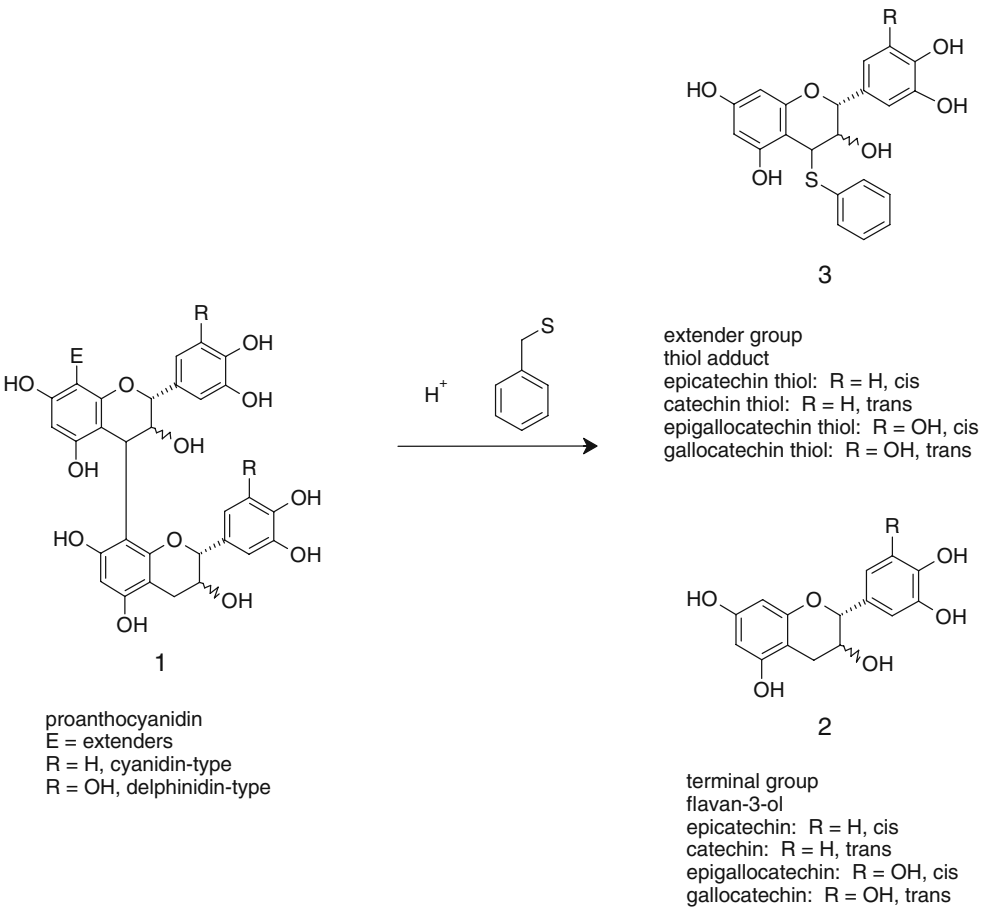


Fig. 1 Proanthocyanidin precursor and thiolysis reaction products. In the presence of acid and benzyl mercaptan, proanthocyanidins (1) react to yield the terminal group as a free flavan-3-ol (2) and the extenders as thiol adducts (3). The dihydroxylated flavan-3-ols (catechin, epicatechin) are characteristic of procyanidins, and the trihydroxylated flavan-3-ols (gallocatechin, epigallocatechin) are characteristic of prodelphinidins

Table 1 Retention times[a] and ms data for flavan-3-ols and thiols produced by reaction of proanthocyanidins with phenylmethanethiol in acidic conditions

Compound	Source	C18 (min)	C8 (min)	[M-H⁻] *m/z*	MS^2 *m/z*
Gallocatechin (2R, 3S)	Commercial	4.0	3.5		
Epigallocatechin (2R, 3R)	Commercial	4.3	4.1		
Catechin (2R, 3S)	Commercial	6.3	5.0		
Epicatechin (2R, 3R)	Commercial	7.0	6.6		
Gallocatechin thiol (2R, 3S)	Aspen proanthocyanidin	12.9	17.3	426.9	302.9
Epigallocatechin thiol (2R, 3R)	Sainfoin proanthocyanidin	12.6	18.3	426.9	302.9
Catechin thiol (2R, 3S)	C2 trimer	15.2	26.3	410.9	287.1
Epicatechin thiol (2R, 3R)	Sorghum proanthocyanidin	14.5	27.6	410.9	287.1

[a] Gradient HPLC with trifluoroacetic acid-modified aqueous and acetonitrile mobile phases was carried out on reversed phase columns with either C18 or C8 stationary phases. Detection was at 220 nm.

360 mV (catechin), 180–300 mV (catechin thiol), 360–480 mV (epigallocatechin thiol), and 240–360 mV (epicatechin thiol). The other flavan-3-ols and thiol adducts were not present in our plant samples, so we did not optimize their electrochemical detection. We confirmed the validity of the EC detection by comparing the compositions determined by EC to the compositions determined by UV for narrowleaf samples that contained large amounts of proanthocyanidins, easily detected with either method.

We converted peak areas obtained from either UV or electrochemical detection to moles using the standard curve, and then calculated moles relative to the moles of terminal flavan-3-ol, giving average composition for the proanthocyanidin from each sample. The sum of the mole ratios provided an estimate of the average degree of polymerization.

Direct thiolysis was carried out on a methanol extract of proanthocyanidins from the dried, ground leaf tissue. It was essential to extract pigments and free flavan-3-ols before extracting proanthocyanidins to prevent interference in terminal unit determination. Plant tissue was extracted first with 2:1 (v/v) chloroform in methanol to remove pigments and then with ethyl acetate to remove free flavan-3-ols. HPLC analysis of the ethyl acetate fractions showed that the characteristic broad peak due to proanthocyanidins was never present in the ethyl acetate fraction, consistent with the poor solubility of proanthocyanidins in ethyl acetate. Methanol extracts of plant tissues not extracted with ethyl acetate contained catechin, but the pre-extraction with ethyl acetate removed all of the free catechin from the tissue so the methanol extracts contained only the broad, unresolved peak characteristic of proanthocyanidins on RP-HPLC.

After thiolysis the products were qualitatively and quantitatively analyzed by HPLC. As expected, thiolysis reaction products did not include the typical proanthocyanidin peak but comprised peaks with retention times consistent with the underivitized flavan-3-ol, catechin; and with the various thiol derivatives of flavan-3-ols. The thiols produced from *Populus* proanthocyanidins included epicatechin thiol and epigallocatechin thiol, with lower levels of catechin thiol. Gallocatechin thiol was rarely detected. There was no evidence for branched proanthocyanidins (4,6 linkages), A-type linkages, or galloyl esters. All of these variations produce thiolysis products that are chromatographically distinct from the standard 4-thiol derivatives of the flavan-3-ols, and all the products from thiolysis reactions of *Poplulus* leaves corresponded to the standard derivatives.

Acid butanol analysis was used to evaluate the distribution of proanthocyanidins between soluble and cell wall-bound fractions in the four cross types. Distribution between soluble and bound forms was consistent across cross types, with less than 10% of the total proanthocyanidin associated tightly with the cell walls in any of the plants. Because there was no quantitative variation in cell wall-bound proanthocyanidins, and because methods for qualitative analysis of cell wall-bound proanthocyanidins have not been developed, we restricted the remainder of our analyses to the soluble proanthocyanidins.

Effect of Genetics on Proanthocyanidin Composition and Chain Length The strongest effect on proanthocyanidin composition was cross type (Table 2). Leaf proanthocyanidins from narrowleaf and backcross were similar in composition (Table 3, Fig. 2). The proanthocyanidins in these two cross types were made up of about 40–50% prodelphinidin-type (epigallocatechin) extenders. A typical narrowleaf proanthocyanidin in June comprises epicatechin$_{11}$-epigallocatechin$_8$-catechin$_2$-catechin$_{(terminal)}$ with less than one gallocatechin per chain (Table 3). In June, backcross proanthocyanidin has a slightly higher degree of polymerization and averages one gallocatechin per chain (Table 3).

The Fremont proanthocyanidin was distinctly different from the proanthocyanidins from the other cross types (Table 3, Fig. 2). In contrast to narrowleaf or backcross proanthocyanidins, the Fremont samples yielded very small

Table 2 Statistical comparisons of genetic, seasonal, developmental and environmental effects on proanthocyanidin composition in *populus*[a]

Effect	DF	Epicatechin		Epigallocatechin		Catechin		Chain Length	
		F	P	F	P	F	P	F	P
Cross type	3,38	28.02	<0.0001	12.88	<0.0001	18.38	<0.0001	19.25	<0.0001
Season[b]	2,54	1.48	0.2371	0.67	0.5168	6.80	0.0023	2.25	0.1147
Cross type × Season	6,54	1.42	0.2248	1.26	0.2901	0.87	0.5214	1.43	0.2205
Developmental zone[c]	1,14	0.54	0.4757	0.65	0.4350	7.86	0.0141	0.21	0.6555
Genotype	4,14	2.17	0.1258	1.23	0.3420	4.72	0.0128	1.39	0.2867
Development × Genotype	4,14	0.73	0.5840	0.84	0.5217	2.62	0.0797	0.77	0.5616
Environment[d]	2,20	0.54	0.5931	0.89	0.4259	3.20	0.0625	0.73	0.4944
Genotype	2,20	4.22	0.0295	0.38	0.6859	8.15	0.0026	0.54	0.5904
Environment × Genotype	4,20	3.89	0.0171	4.50	0.0094	0.44	0.7756	4.35	0.0108
Garden (Backcross clone 10)	2,9	0.26	0.7739	0.57	0.5845	0.50	0.6226	0.70	0.5197

[a] Effects were not tested for gallocatechin, which was above the limit of detection only in some narrowleaf and backcross samples from June 2003.

[b] Samples of each cross type collected in late May, late June, and late July 2003 in the ONC common garden.

[c] Samples were collected in June 2007 from both the juvenile zone and the mature zone of four trees each from five narrowleaf genotypes in the ONC common garden.

[d] Samples were collected in June 2007 from mature branches of the same genotypes planted at three different common garden locations: North Pit, ONC and Taggart gardens.

amounts of the prodelphinidin type subunits (epigallocatechin, gallocatechin). Furthermore, the Fremont proanthocyanidins were short chain, so typically Fremont yielded dimers comprised of an epicatechin or catechin extender with catechin terminal.

The composition of the proanthocyanidins from the F1 cross type was intermediate to the narrowleaf and Fremont parents but was more similar to Fremont (Table 3, Fig. 2). Like Fremont, the proanthocyanidin from F1 cross types was comprised primarily of procyanidin-type subunits. Chain lengths were shorter than narrowleaf but are longer than Fremont. A typical composition for leaves collected in June is epicatechin$_4$-catechin$_2$-catechin$_{(terminal)}$ with less than one epigallocatechin per chain (Table 3).

Effect of Season, Developmental Zone, and Environment on Proanthocyanidin Composition and Chain Length Season had little effect on proanthocyanidin composition (Table 2, Fig. 2). For each cross type, tissue samples collected in May, June, and July from the ONC common garden exhibited no differences in the major proanthocyanidin subunits epicatechin or epigallocatechin, or in chain length. There were more statistically significantly catechin extenders in June than in May or July in narrowleaf, backcross and F1 crosstypes, but the chemical significance of the difference is questionable, since the change is less than one catechin extender per average chain (Table 2, Fig. 2). The rare subunit gallocatechin was detected only in samples from June, and only in narrowleaf and backcross plants. We conclude that proanthocyanidin composition in *Populus* changes little over the growing season, and that any effects are limited to the less common flavan-3-ol subunits.

Developmental zone did not affect the major proanthocyanidin subunits epicatechin or epigallocatechin, or chain length, in the narrowleaf crosstype (Table 2, Fig. 3). The only effect of developmental zone was limited to the

Table 3 Composition and chain length of proanthocyanidins in four cross types of *populus*[a]

Cross type (n)	Epicatechin (SEM)		Epigallocatechin (SEM)		Catechin (SEM)		Gallocatechin (SEM)		Chain Length (SEM)[b]	
Narrowleaf (9)	11.3	(3.0)	7.6	(2.4)	1.6	(0.3)	0.5	(0.3)	21.9	(5.6)
Fremont (10)	0.4	(0.1)	0.2	(0.1)	0.4	(0.1)	nd[c]		1.9	(0.1)
F1 (8)	4.4	(1.3)	0.4	(0.4)	1.5	(0.3)	nd		7.3	(1.8)
Backcross (10)	16.1	(4.2)	15.6	(6.2)	1.5	(0.3)	1.1	(0.5)	35.3	(10.6)

[a] Samples were collected in late June of 2003. Composition is in moles relative to the terminal unit for all proanthocyanidins, catechin and is expressed as the average and the standard error of the mean (SEM).

[b] Chain length is calculated as the sum of the extenders plus one terminal catechin.

[c] *nd* Not detected.

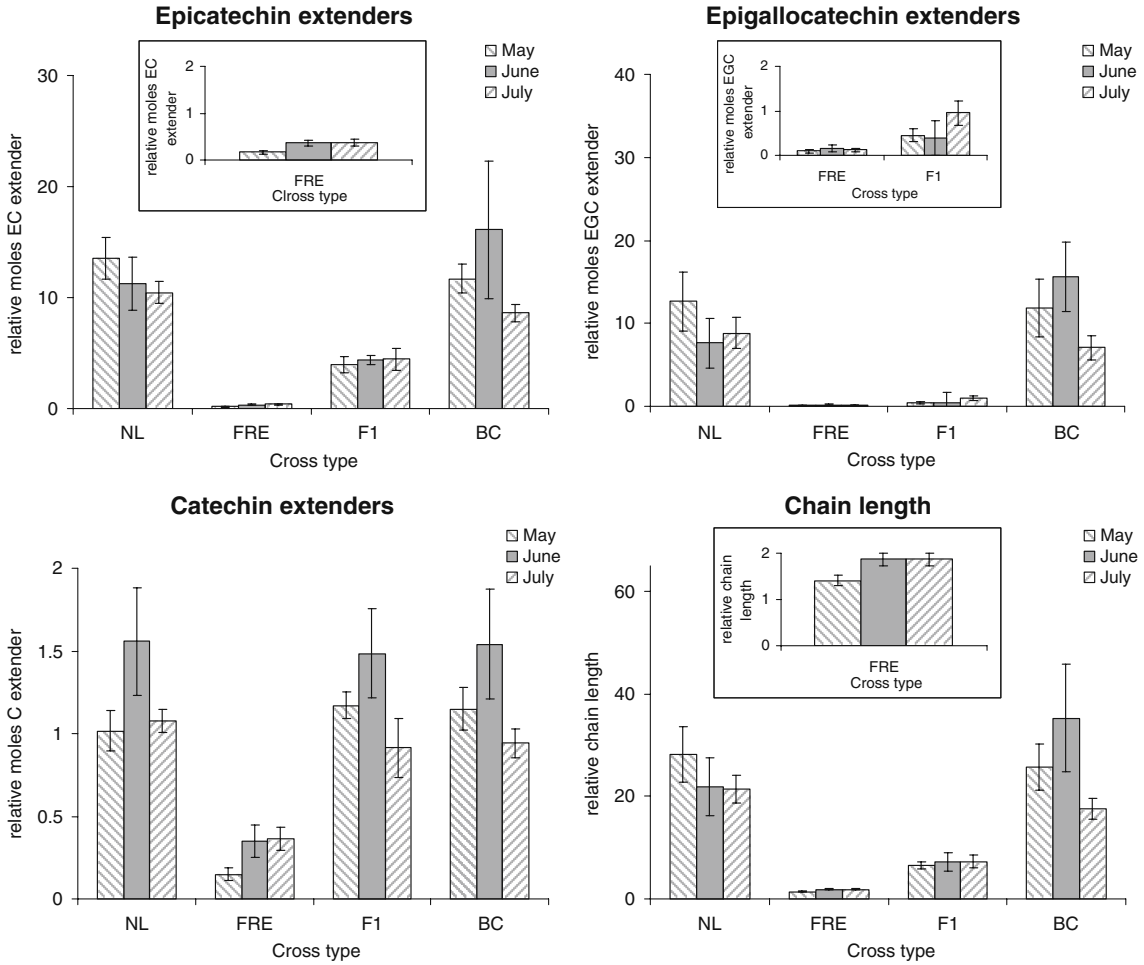


Fig. 2 *Populus* proanthocyanidin composition and chain length as a function of season. Samples of mature leaves were collected from four crosstypes [narrowleaf (NL), Fremont (FRE), F1 and backcross (BC)] growing in the ONC garden center in late June, late July and late August 2003. *Bars* show mean for N=7–10, error bars are 1 SEM

relatively uncommon subunit catechin, which was more abundant in proanthocyanidins from mature tissue than from juvenile tissue in all five genotypes (Table 2, Fig. 3). We conclude that reproductive maturity in *Populus* does not change the major foliar tannin composition.

Distinct environmental conditions found in the three gardens led to an environment × genotype effect on proanthocyanidin composition in three clones of narrowleaf, but had no effect on one clone of backcross (Table 2, Fig. 4). Although trees in the three gardens were of different ages, the dominant differences between the gardens were most likely environmental and not age-related. Trees in all three gardens were at least 15 years old, and previous work with the related *Populus tremuloides* revealed that age-related variation in leaf chemistry in trees of this age is minimal (Donaldson et al., 2006). The largest effect of environment was on narrowleaf clone 1019, which produced much shorter chain length proanthocyanidins in the ONC garden than in the NP or T gardens. In ONC plants, this proanthocyanidin had an average composition of epicatechin$_3$-epigallocatechin$_2$-catechin$_1$-catechin$_{(terminal)}$ with less than one gallocatechin per chain.

Discussion

In previous studies of proanthocyanidin accumulation in cross types of *Populus*, the proanthocyanidins were examined quantitatively but not qualitatively. For example, Rehill et al. (2006) found important quantitative differences in proanthocyanidins among cross types. Relatively large amounts of proanthocyanidin were found in leaves from backcross and narrowleaf, while Fremont and F1 accumulated significantly smaller amounts of proanthocyanidins. Our study confirmed those quantitative differences. Proanthocyanidins in both backcross and narrowleaf were easily

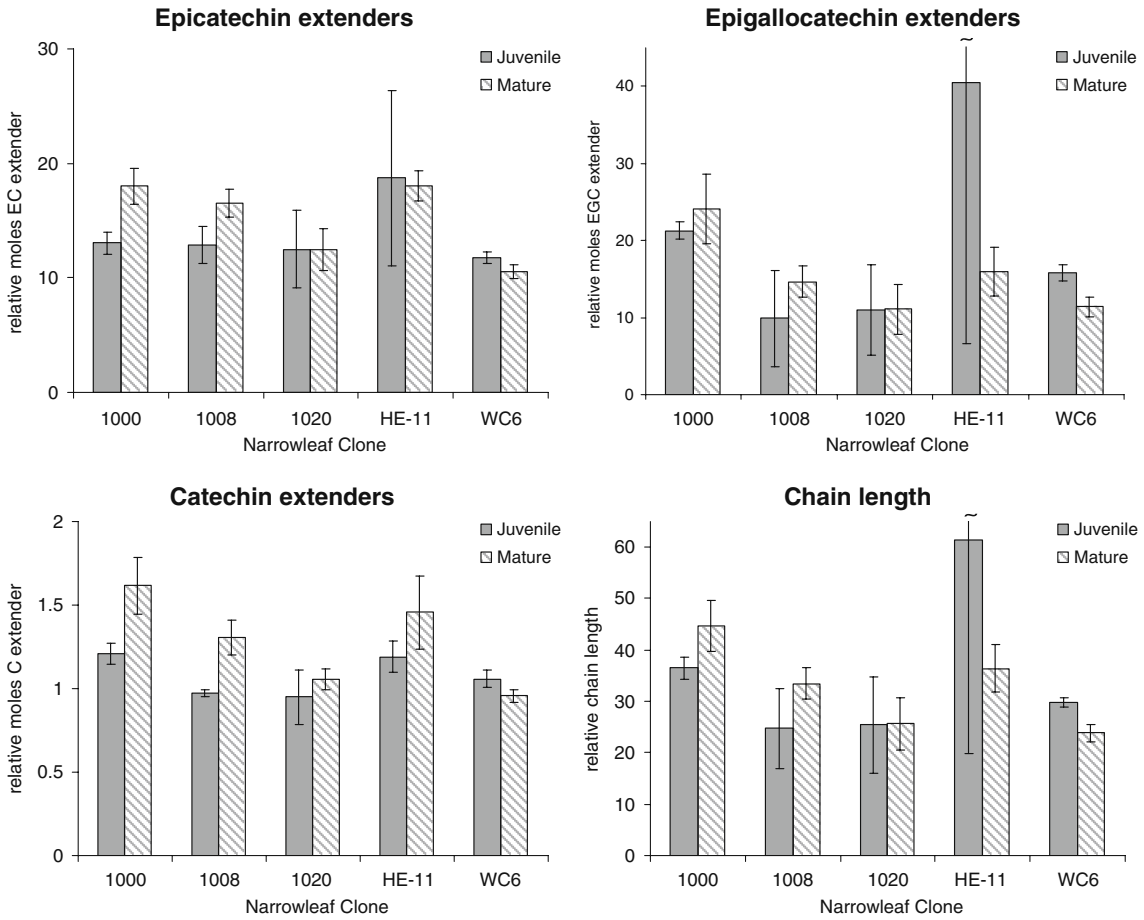


Fig. 3 *Populus* proanthocyanidin composition and chain length as a function of developmental zone. Samples of juvenile or mature leaves were collected from the same trees for five clones of narrowleaf growing in the ONC garden center in June 2007. *Bars* show mean for $N=4$, error bars are 1 SEM. Upper error bars are truncated for epigallocatechin and chain length

analyzed by thiolysis with UV-HPLC detection. Leaf tissue from F1 genotypes contained far less proanthocyanidin, but UV-HPLC analysis was still possible. Fremont contained very low levels of proanthocyanidin, and the thiolysis products could only be analyzed with the additional sensitivity afforded by EC detection.

Acid butanol analysis of soluble and cell wall-bound proanthocyanidins indicated that the quantitative differences in extractible tannins reflect differences in total proanthocyanidins, not differences in subcellular localization of proanthocyanidins. In all cross types there is little cell wall-bound proanthocyanidin. Analyses of methanol-soluble proanthocyanidins by acid butanol assays (Rehill et al., 2006) or by thiolysis (this paper) provide reliable information for *Populus* leaf tissue.

Our study expands and elucidates the qualitative description of proanthocyanidins reported for *P. balsamifera* and *P. tremuloides* by Ayres et al. (1997). These species contained mixed condensed tannins, with procyanidin (dihydroxylation, see Fig. 1) dominating the *P. balsamifera* tannin and prodelphinidin (trihydroxylation, see Fig. 1) dominating in *P. tremuloides*. We found that *P. angustifolia* also contains mixed procyanidin-prodelphinidin condensed tannin with roughly equal amounts of the two types of subunits. However, *P. fremontii* contains a simple procyanidin made up of epicatechin- and catechin-type monomers. The F1 cross type in our study predominantly accumulates the procyanidin-type flavan-3-ols, but the effect of the narrowleaf parent is obvious in the slightly elevated level of the trihydroxylated epigallocatechin compared with Fremont. The backcross cross type is more similar to the narrowleaf, producing a mixed procyanidin-prodelphinidin tannin with neither type of subunit dominating the composition.

A highly variable qualitative feature of the *Populus* cross types is proanthocyanidin chain length. The proanthocyanidins from narrowleaf are polymers with 20–28 flavan-3-ol subunits, while the Fremont proanthocyanidin is a dimer. The F1 crosses of Fremont × narrowleaf are oligomers of six to seven subunits, making them more similar to Fremont than to narrowleaf. The proanthocyanidin chain length for backcross genotypes are polymers with 17–35 subunits, similar but more variable than the narrowleaf parent.

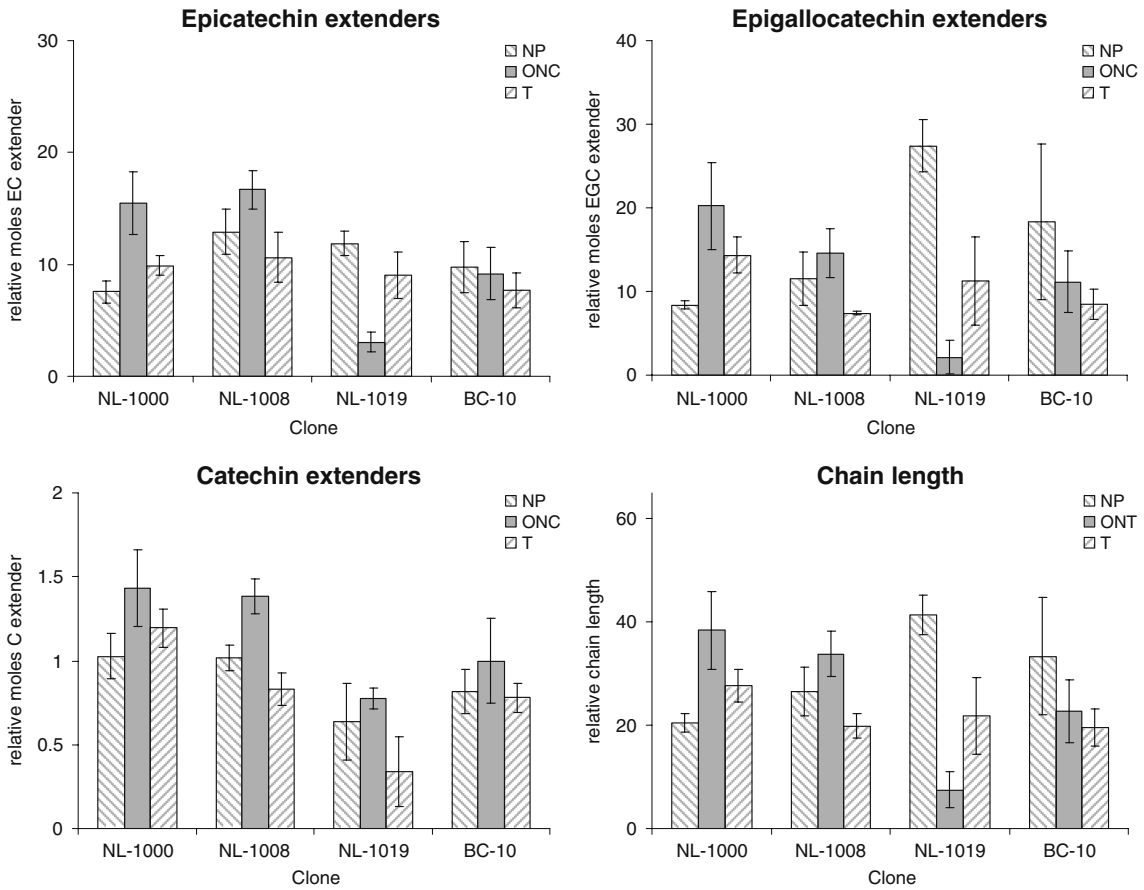


Fig. 4 *Populus* proanthocyanidin composition and chain length as a function of environment. Samples of mature leaves were collected from three five clones of narrowleaf (NL) or one clone of backcross (BC) in June 2007. Plants were growing in the North Pit (NP), the Ogden Nature Center (ONC), or the Taggart (T) common garden. Bars show mean for N=2–5, error bars are 1 SEM

Our work suggests that qualitative attributes such as proanthocyanidin composition and chain length may be controlled by dominant inheritance in both F1 and backcross cross types. This result is partly consistent with reports that quantity of proanthocyanidins is controlled by dominant inheritance in backcrosses (Driebe and Whitham, 2000), but contrasts with some other reports of additive inheritance for proanthocyanidin levels (Rehill et al., 2006). The heritability of phenolic glycoside composition in *Populus* is controlled via dominance (Rehill et al., 2006) but flavonoid composition appears to involve additive control at the F1 generation and dominance in backcrosses (Crawford, 1974). Further studies on the mode of inheritance of qualitative traits such as proanthocyanidin composition and chain length could be initiated by using methods of chemical analysis similar to those we used here.

The effect of environment on narrowleaf proanthocyanidins differed among genotypes (environment × genotype interaction). The strongest effect was for narrowleaf clone 1019: in the ONC garden, proanthocyanidin chain length for genotype 1019 (7.5) was similar to that of F1 plants (7.0). However, the importance of the prodelphinidin-type subunit in the clone 1019 proanthocyanidin was typical of narrowleaf, not F1 plants. It was surprising that this difference was noted only in the ONC garden, which has environmental conditions most similar to the NP garden.

Our study did not identify any strong effect on qualitative characteristics of proanthocyanidins due to season or developmental zone. Season and developmental zone statistically influenced the minor proanthocyanidin subunit catechin, but the difference is not chemically meaningful. Although catechin is elevated slightly in tissue collected in June or in mature tissue, the chain length is not significantly different for either season or developmental zone, indicating that the change in catechin is not chemically significant. This result contrasts with quantitative studies, in which tissue developmental zone had a strong effect on the amount of proanthocyanidins in *Populus,* with mature tissue containing about twice as much tannin as juvenile tissue (Rehill et al., 2006). In *Populus*, quantitative and qualitative expression of proanthocyanidins are controlled independently, at least in part.

Qualitative traits that vary may provide insight into metabolic capabilities and regulatory steps in cases where

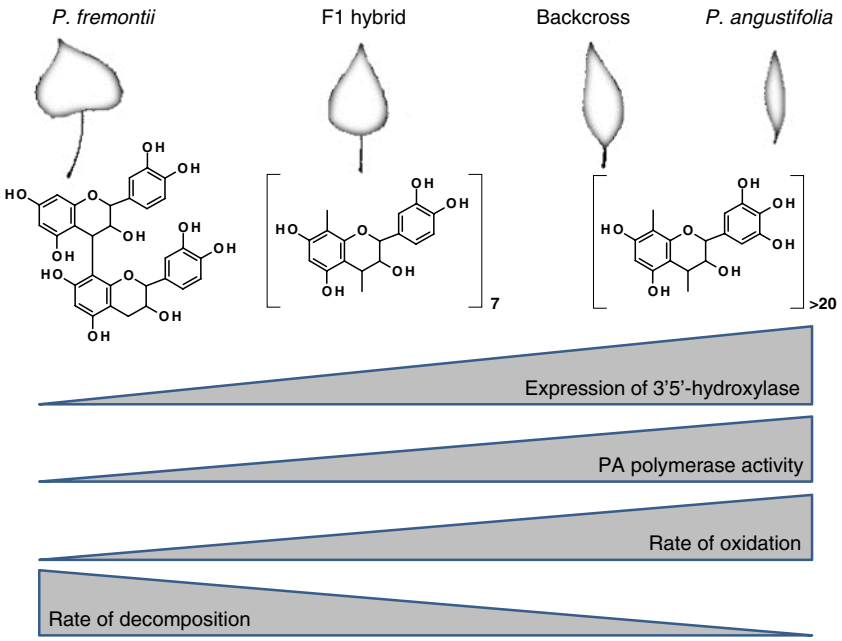


Fig. 5 Relationships among the plants, the qualitative features of the proanthocyanidins, and biochemical and ecological hypotheses. The leaf icons represent the parent species *Populus fremontii* and *Populus angustifolia*, the F1 hybrid and the backcross. Under each leaf is a structural formula summarizing type of subunit (procyanidin or prodephinidin) and chain length typical of the proanthocyanidin found in that plant. The gray triangles depict the proposed metabolic and ecological trends, with the large end of the triangle representing high level of 3′5′-hydroxylase expression, leading to prodelphinidin subunits; high level of putative proanthocyanidin polymerase activity, resulting in longer chain lengths; and rapid oxidation or decomposition in the environment, predicted based on chemical reactivity and tentatively confirmed by work from Barbehenn et al., 2006 and Driebe and Whitham, 2000

the fundamental metabolic processes are known. For proanthocyanidins, differences in monomer composition presumably reflect flavonoid metabolism, while differences in chain length reflect aspects of the polymerization steps. The biosynthetic steps leading to the monomeric precursors of the proanthocyanidins are well defined. Some of the most detailed studies of the pathway have been conducted with *Populus* (Tsai et al., 2006). Despite many efforts to elucidate the final steps of proanthocyanidin synthesis, the exact details of the polymerization steps are unknown.

Proanthocyanidin composition is determined by the precursors supplied by the metabolic grid (network of pathways) characteristicof flavonoid production (Stafford, 1990). In *Populus*, the differences between cross types involve B-ring hydroxylation. Narrowleaf proanthocyanidins contain both dihydroxylated (3′4′-hydroxyl) and trihydroxylated (3′4′5′-hydroxyl) subunits, while Fremont is restricted to the dihydroxylated flavan-3-ol units. B-ring hydroxylation is carried out by two enzymes, flavonoid 3′-hydroxylase (CYP75B) and flavonoid 3′,5′-hydroxylase (CYP75A) (Ayabe and Akashi, 2006). Both enzymes are cytochrome P450 monooxygenases that use NADPH, but sequence and mechanistic studies have indicated that the two enzymes diverged early (Seitz et al., 2007). The *Populus* genome is reported to have one copy of the flavonoid 3′-hydroxylase gene and two duplicated flavonoid 3′,5′-hydroxylase genes (Tsai et al., 2006), so cross type differences in proanthocyanidin composition may reflect genetically dictated differences at the expression level.

In tissue that does not express the flavonoid 3′,5′-hydroxylase, the trihydroxylated prodelphinidin- type flavonoids are not produced. Our data suggest that Fremont does not express the flavonoid 3′,5′-hydroxylase gene but that narrowleaf does produce that enzyme in sufficient quantity to make the prodelphinidin-type proanthocyanidins. Chemosystematic studies that focus on flavonoid metabolism support that hypothesis. For example, Crawford (Crawford, 1974) reported that an important chemical difference between narrowleaf and Fremont was the accumulation of myrecetin, a trihydroxylated flavonol glycoside, in narrowleaf but not in Fremont. Only 3′-hydroxylated or 3′,4′-hydroxylated flavonoids were found in Fremont, consistent with the absence of flavonoid 3′,5′-hydroxylase from that cross type. We found that F1 hybrids of narrowleaf and Fremont accumulated proanthocyanidins with some prodelphinidin-type subunits, so we speculate that low levels of the enzyme must be expressed in the hybrid tissue. Molecular studies support that perspective, with RT-PCR analysis of an F1 hybrid indicating that flavonoid 3′,5′-hydroxylase is expressed in leaf tissue of a F1 hybrid (Tsai et al., 2006). An examination of the expression of the two hydroxylases in narrowleaf, Fremont,

and backcross leaf tissue would provide an additional test of our hypothesis that composition of proanthocyanidins in *Populus* reflects the levels of flavonoid 3′,5′-hydroxylase.

Proanthocyanidin chain length may reflect either differences in substrate supply or differences in the expression of a polymerizing enzyme. Limited production of chain extenders, or overproduction of terminal units, would result in production of short chain proanthocyanidins. Proanthocyanidin substrate supply is controlled by the flavonoid pathway. Although the details of polymerization are not well established, flavan-3-ols are the most likely chain elongation units (Gupta and Haslam, 1978). Flavan-3-ols are produced by action of flavanone 3-hydroxylase on the central metabolite naringenin (Tsai et al., 2006). We hypothesize that proanthocyanidin chain length in Fremont is limited by supply of substrate due to absence of flavanone 3-hydroxylase. That hypothesis is supported by surveys of flavonoid production that showed that Fremont accumulated flavones and flavanones, but not flavonols (Crawford, 1974; Greenaway et al., 1989). In contrast, narrowleaf accumulates flavonols as the predominant flavonoid (Crawford, 1974), consistent with expression of flavanone 3-hydroxylase, and consistent with abundant substrate for production of polymeric proanthocyanidins. Since F1 hybrids produce oligomeric proanthocyanidins, we postulate that this cross type expresses only low levels of the flavanone 3-hydroxylase, and that notion is confirmed by expression studies in which weak expression of flavanone 3-hydroxylase was reported in leaf tissue of an F1 hybrid (Tsai et al., 2006). Additional gene expression studies in all four cross types would test this hypothesis more completely.

An alternative explanation for the production of short chain proanthocyanidins in Fremont would be absence of the enzyme responsible for polymerization. Unfortunately, the chain elongation step of proanthocyanidin biosynthesis has not been characterized for any system (Xie and Dixon, 2005). We suggest that gene expression studies of closely related cross types that produce proanthocyanidins with very different chain lengths, such as narrowleaf, Fremont, and F1 hybrid *Populus*, might provide useful leads to the elusive polymerase enzyme.

We restricted our analyses of *Populus* proanthocyanidin composition to leaf tissue. However, in grapes, production of proanthocyanidins is tissue-selective, with skins producing mixed procyanidin/prodelphinidin tannins and seeds producing only procyanidin (Monagas et al., 2003) There is little information on proanthocyanidins from other tissues of *Populus*, but tissue-specific expression of the flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase have been reported (Tsai et al., 2006). Based on that information, we predict that proanthocyanidins from the root tips of F1 may comprise only cyanidin-type subunits, since RT-PCR suggests that this tissue does not have the enzyme required to produce the trihydroxylated precursors to prodelphinidin-type subunits (Tsai et al., 2006). Tissue-selective expression of proanthocyanidins needs to be more fully explored using tools that elucidate qualitative differences.

The idea that "tannins", broadly encompassing both proanthocyanidins and hydroyzable tannins, have a quantitative effect on ecosystem processes has been a cornerstone of chemical ecology for decades (Feeny, 1976). Despite many refinements to the original ideas, most studies treat tannins strictly quantitatively. Nonspecific quantitative methodology and limited qualitative analysis are the norms for studies that include consideration of tannins. For example, the rates of litter decomposition for *Populus* cross types were correlated with levels of proanthocyanidins (Driebe and Whitham, 2000). Leaves from Fremont decomposed quickly (0.007 day^{-1}), backcross decayed two- to three-times more slowly (0.17 day^{-1}), and narrowleaf and F1 decomposed at an intermediate rate (0.012 day^{-1}). For Fremont and narrowleaf, there appeared to be a strong relationship between rate of decomposition and level of proanthocyanidin, supporting the generally held view that levels of proanthocyanidins dictate biological activity. However, a more complex relationship was revealed by the data from narrowleaf and F1 hybrids. Leaves from these two cross types decomposed at the same rate even though levels of proanthocyanidins were four- to five-times higher in narrowleaf crosstypes than in F1 hybrids. We propose that the qualitative traits of subunit composition and polymer chain length of proanthocyanidins are important determinants of bioactivities such as decomposition rate. For example, comparing the results of our study to the litter decomposition studies, we see that both prodelphinidin subunit composition and proanthocyanidin chain length are inversely correlated to the rate of decomposition, with the cross type we identified as having the longest chain length and highest% prodelphinidin subunit (narrowleaf) also having the slowest decomposition. Nierop et al. (2006) noted that prodelphinidins slowed N mineralization rates more than procyanidins in Corsican pine litter amended with structurally well defined condensed tannins. Litter decomposition was negatively correlated with degree of polymerization of condensed tannins for several species of tropical trees, but the correlation was strongly driven by a single species containing very highly polymerized tannins (Coq et al., 2010). Explicit tests that use well characterized, diverse tannins, of the relationship between condensed tannin structure and rate of litter decomposition are needed

Activities other than rate of decomposition of leaf litter also may be significantly affected by the qualitative characteristics of proanthocyanidins. For example, Barbehenn et al. (2006) demonstrated that polymeric prodelphinidins were more susceptible to oxidative browning than lower

molecular weight prodelphinidins. In the same study, procyanidins were more resistant to oxidation than prodelphinidins. Oxidative browning and radical formation might be important determinants of toxicity and metabolic fate of proanthocyanidins in insects with alkaline gastrointestinal conditions, leading to the conclusion that proanthocyanidin qualitative traits might govern the role of these compounds in plant-herbivore interactions.

Our work demonstrates that in *Populus* leaf tissue, genetics (i.e., cross type) is the primary determinant of proanthocyanidin quality—both chain length and monomer composition. Environment has a less important role in determining proanthocyanidin quality, and developmental zone and season are much less important. In contrast, the quantity of proanthocyanidin is influenced by developmental factors as well as genetics and environment (Rehill et al., 2006). The variation in proanthocyanidin composition and chain length found in these four well-characterized *Populus* cross types suggest that this hybridizing complex might serve as an excellent model for examining the functional role of proanthocyanidin quality more carefully (Fig. 5). The cross types also may provide an excellent system for further exploration of the enzymology of proanthocyanidin polymerization, and for probing the metabolic regulation of proanthocyanidin composition (Fig. 5).

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Foliage Chemistry Influences Tree Choice and Landscape Use of a Gliding Marsupial Folivore

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Abstract The chemical quality of forage may determine landscape use and habitat quality for some herbivorous species. However, studies that investigate the relationship between foliar chemistry and foraging choices in wild vertebrates are rare. *Petauroides volans* (the greater glider) is unique among Australian marsupial folivores because it glides. It also frequently consumes foliage from both major *Eucalyptus* subgenera, *Eucalyptus* (common name "monocalypt") and *Symphyomyrtus* (common name "symphyomyrtle"), which differ markedly in their foliar chemistry. Such differences are thought to be a product of co-evolution that also led to guild-specific plant secondary metabolite (PSM) specialization among other marsupial eucalypt folivores. To explore whether foliar chemistry influences tree use, we analyzed foliage from eucalypt trees in which we observed *P. volans* during a radio tracking study and from eucalypt trees in which animals were never observed. We used a combination of chemical assays and near infrared spectrophotometry (NIRS) to determine concentrations of nitrogen (N), *in vitro* available nitrogen (AvailN), and *in vitro* digestible dry matter (DDM) from foliage sampled from the monocalypt and symphyomyrtle species, and total formylated phloroglucinol compounds (FPCs) and sideroxylonals (a class of FPCs) from the symphyomyrtle species (FPCs do not occur in monocalypts). Tree size and spatially-dependent, intraspecific variations in sideroxylonals and DDM concentrations in the symphyomyrtle foliage and of N, AvailN, and DDM in the monocalypt species were important indicators of tree use and habitat suitability for *P. volans*. The results i) demonstrate that guild-specific PSMs do not always lead to guild-specific foraging; ii) provide a compelling co-evolutionary case for the development of gliding in *P. volans*; and iii) have implications for the management and conservation of this and other folivorous species.

Key Words Herbivory · Available nitrogen · Plant secondary metabolite · Plant-animal interaction · *Eucalyptus* · *Petauroides volans* · Specialist · Coevolution

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Introduction

Variations in plant nutrients and herbivore-deterrent plant secondary metabolites (PSMs) across a landscape create a chemically complex environment that may influence the distribution and abundance of animal species (Braithwaite et al. 1983; Stephens and Krebs 1986; Moore et al. 2010). Identifying the factors that determine habitat suitability is essential for the conservation of species and communities. Those species that survive in a relatively narrow niche, both in terms of the range of food-types available and the variation of chemical composition that they can tolerate, may be particularly sensitive to landscape and climatic changes that can affect the quality and availability of their food (Lawler et al. 1997; Moore et al. 2004).

Eucalyptus (L'Herit) foliage contains relatively low concentrations of nutrients, particularly nitrogen (N), but high concentrations of PSMs that can cause toxicosis and reduce the digestibility of plant nutrients (Cork and Foley 1991; Moore et al. 2004; Scrivener et al. 2004). Concentrations of

nutrients and PSMs in eucalypts often vary considerably from tree to tree, even within the same species (Wallis et al. 2002; Andrew et al. 2005). Several studies have demonstrated how variation in N, tannins, and formylated phloroglucinol compounds (FPCs) differentially affect feeding by *Phascolarctos cinereus* (the koala), *Pseudocheirus peregrinus* (the common ringtail possum), and *Trichosurus vulpecula* (the common brushtail possum) (Lawler et al. 1998a; Wallis et al. 2002; Marsh et al. 2003a, b; Moore and Foley 2005; Moore et al. 2005). It is not known whether intraspecific variations in FPCs or N availability influence the foraging of *Petauroides volans* (the greater glider, Fig. 1), a marsupial folivore that, like *P. cinereus*, feeds almost exclusively on eucalypt leaves.



Fig. 1 The greater glider (*Petauroides volans*), shown here in light (**a**) and dark (**b**) color phases, is a marsupial folivore that feeds primarily on *Eucalyptus* leaves. Photographs by David Lindenmayer

Differential susceptibility to PSMs may reduce competition among co-occurring herbivores by restricting them to eating plants that contain the unique chemical signature they have evolved to tolerate (Moore et al. 2004). It may be appropriate to consider leaf-eating animals, not as folivores *per se,* but as specialists on particular metabolites. Determining the PSM tolerances of co-occurring herbivorous species also could shed light on evolutionary relationships between plants and animals and current patterns of species distributions and biodiversity in particular habitats.

In addition to intraspecific variation in foliar chemistry, there are also differences in chemistry among eucalypt species in the two major eucalypt subgenera, *Eucalyptus* (common name "monocalypt") and *Symphyomyrtus* (common name "symphyomyrtle"). Symphyomyrtle species produce FPCs that, when ingested by mammals, stimulate the serotonergic systems and probably induce sensations of nausea (Lawler et al. 1998b). When feeding on eucalypts, both *P. cinereus* and *T. vulpecula* feed mainly on symphyomyrtle species. Although tolerant of FPCs, captive animals eat less as foliar concentrations of these compounds increase (Lawler et al. 1998a; Wallis et al. 2002). Similarly, FPCs, including sideroxylonals, influence tree use by free-living *T. vulpecula* and *P. cinereus* (Scrivener et al. 2004; Moore and Foley 2005; Moore et al. 2010). In contrast, *P. peregrinus* is relatively intolerant of FPCs and feeds primarily on monocalypt species (Lawler et al. 1998a; Moore et al. 2004).

Monocalypts do not produce FPCs (Eschler et al. 2000) but their foliage contains high concentrations of tannins (Wallis et al. 2010) that can bind to nitrogenous compounds in plant tissues and reduce the digestibility of plant proteins (Silanikove et al. 1996; Marsh et al. 2003b). Using an *in vitro* assay (DeGabriel et al. 2008), Wallis et al. (2010) showed that the foliage of 31 monocalypt species contained about half the available nitrogen (AvailN) of that of 82 symphyomyrtle species due to N binding by tannins. Tannins influence the physiology and foraging behavior of many herbivorous species, although not all marsupial folivores appear to be affected equally (Osawa and Sly 1992; Silanikove et al. 1996; Marsh et al. 2003a). When tannins are inactivated by coating leaves with polyethylene glycol (PEG), some herbivores, including *T. vulpecula*, eat more of the treated material, while others, such as *P. peregrinus*, do not (Marsh et al. 2003b). DeGabriel et al. (2009) showed that N availability in eucalypt foliage is an important determinant of reproductive success for *T. vulpecula* in the wild.

In mixed stands of eucalypt trees, representatives from both major subgenera tend to co-dominate and this is known as Pryor's Rule (Pryor 1959). Thus, one can suggest that by specializing on the PSMs produced by a particular eucalypt subgenus, folivores could inhabit the same tracts

of forest without competing for food (Marsh et al. 2003a). In turn, coevolution between plants and animals may have influenced the diversity of plant chemotypes and foraging adaptations of herbivores (Ehrlich and Raven 1964; Thompson 1994; Futuyma 2000; Becerra 2007). As a result of such co-evolutionary relationships, guild-specific PSMs are often thought to lead to guild-specific foraging (Thompson 1994; Rausher 2001).

We focused on *P. volans*, the only Australian folivorous marsupial that glides. Although past studies have linked foliar concentrations of N and tannins to tree species preference and forage consumption (Foley and Hume 1987; Kavanagh and Lambert 1990; McIlwee et al. 2001), we do not know how this animal responds to variation in the concentrations of AvailN or FPCs. Although *P. volans* is argued to be a monocalypt and tannin specialist, like the closely related *P. peregrinus* (Family *Pseudocheiridae*) (Moore et al. 2004), field studies have indicated that it regularly consumes foliage from eucalypt species of both major subgenera (Kavanagh and Lambert 1990; Cunningham et al. 2004).

We posed a series of questions to investigate the role of eucalypt foliage chemistry on tree choice, landscape use, and the habitat requirements of *P. volans*. Due to the association of particular PSMs (e.g., FPCs) with particular eucalypt subgenera, the first three questions were investigated on the monocalypt and symphyomyrtle species independently. 1) Are there significant differences in foliar concentrations of FPCs (total FPCs and sideroxylonals), N, AvailN, and digestible dry matter (DDM) among trees in which *P. volans* was and was not observed? 2) Is the relationship between *P. volans* tree use and foliar concentrations of N, AvailN, and DDM the same for trees from both major subgenera of *Eucalyptus*? 3) Are feeding-specific observations or spatial considerations (i.e., the distance of trees with no animal observations to trees with observations) important for determining whether there is a relationship between foliage chemistry and tree use? 4) Do our results agree with current hypotheses about the foraging niche of *P. volans* as a "specialist" folivore? 5) Do our results have implications for the conservation of *P. volans* and other folivorous species?

Methods and Materials

Study Area Our study site was 100 km west of Canberra in Buccleuch State Forest, New South Wales, Australia 148° 40′ E, 35°10′ S. The area includes a 50,000 ha exotic pine (*Pinus radiata)* plantation bordered by native eucalypt forest and grazing farmland. Extensive areas of native forest were cleared from the late 1930s through the 1980s to establish the pine plantation, leaving patches of remnant eucalypt forest (1–100 ha) embedded within the pine (Lindenmayer et al. 1999). Five neighboring eucalypt forest remnants were selected for a separate radio-tracking study to investigate *P. volans* home-range size and tree use in a fragmented forest ecosystem (Pope et al. 2004). These remnants ranged in size from 1.6 to 18.2 hectares and varied in shape, but they had similar vegetation communities dominated by narrow-leaved peppermint [*Eucalyptus radiata* (monocalypt)], ribbon gum [*E. viminalis* (symphyomyrtle)] and mountain gum (*E. dalrympleana* [symphyomyrtle)]. A more complete description of the study area, including a list of common canopy and understory vegetation is in Lindenmayer et al. (1999).

Radio-tracking Forty *P. volans* from the five neighboring eucalypt forest remnants were captured and fitted with single-stage radio-transmitter collars (Faunatech Pty Ltd, Victoria, Australia). Effort was taken to ensure that every *P. volans* in the selected remnants was included in the study (see Pope et al. 2004 for details). Tracking occurred over five consecutive nights each month from September 1997 to October 1998. Animals were tracked to one tree per night, and the tracking sequence was varied randomly (for a description of tracking methodology see Cunningham et al. 2004). Once we located a *P. volans*, we recorded its behavior (feeding, grooming, perching, mating, and not observable), tagged the tree in which it was observed, and recorded the tree location with a differentially-corrected, hand-held GPS (Global Star Pty Ltd, Melbourne).

Tree-leaf Sampling Radio-tracking data provided a unique opportunity to investigate tree use by *P. volans* in relation to recently identified PSMs and a newly described measure of forage quality (available nitrogen). A study published on tree use by wild *P. cinereus* based on tree location data recorded from 1993 to 2004 established the feasibility of sampling foliage from feeding trees identified during earlier tracking efforts (Moore et al. 2005). No large-scale changes to the selected remnant areas, such as harvesting of the surrounding pines, which might alter the concentrations of foliar chemistry, have occurred since the original radio-tracking.

In December 2006, we revisited the five fragments and relocated 261 of the original 433 trees used at night time by *P. volans*. Lost tree tags made it impossible to identify all trees from the original study. We collected leaves from all tree species in which *P. volans* was observed (see Cunningham et al. 2004 for a complete list). The vast majority of observations occurred in *E. radiata* (41% of eucalypt tree use) and *E. viminalis-E. dalrympleana* (35%) (Cunningham et al. 2004; Pope et al. 2004). Due to the relatively small number of observations in other species, we focused our analysis solely on these three tree species. Data for *E.*

viminalis and *E. dalrympleana* were pooled during the radio-tracking study due to the difficulty of distinguishing these closely related trees in the field. Both species produce FPCs, and both are eaten by *P. volans*, so grouping them also was appropriate for this study.

We collected foliage from one or more untagged neighboring trees of the same species and similar size [±10% diam. at breast height (DBH)] as each numbered tree (N=112). Cunningham and others (2004) found a correlation between tree size and tree use by *P. volans*, so we selected similar-sized trees to minimize the effect of this potentially confounding factor. If a tree did not have a suitable neighbor within 20 m, we did not sample a neighbor. Where possible, we also sampled canopy trees from the few areas within the remnant patches where no *P. volans* were observed. These trees were more than 20 m from any other tree with an animal observation (N=53). The purpose of this sampling strategy was to document the chemical composition of eucalypts within the proximity of the animals' home-ranges without introducing a potentially confounding distance factor. The aim of the original radio-tracking study was to determine home-range size rather than total tree use (Pope et al. 2004). Animals invariably used trees in addition to those in which they were observed. We recognize that *P. volans* may have used some of the "no observations" trees, though we assumed a bias towards a non-use status for trees in which we did not observe an animal.

We obtained leaf samples by shooting a branch from the top half of the canopy using a 0.222 caliber rifle, and then recorded tree height and DBH at 1.3 m from the forest floor on the uphill side of the tree by using a laser clinometer and a measuring tape, respectively. We collected about 50 g of fully expanded, healthy adult leaves from each tree. *Petauroides volans* feeds on both young and mature foliage but we chose mature foliage because concentrations of both N and PSMs can change considerably as a leaf ages (Choo et al. 1981; Kavanagh and Lambert 1990). Thus, selecting mature foliage—that available for most of the year—ensured that leaf phenology did not obscure the relative concentrations of compounds between trees. We placed the leaf samples in a portable freezer (Engel MT17: Brisbane, Australia) and transported them to the laboratory where they were freeze-dried and then ground to pass a 1 mm sieve using a Cyclotec 1093 mill (Tecator, Sweden).

NIR Spectrometry We placed samples of freeze-dried, ground leaf in an oven (40°C) for 12 h to equilibrate atmospheric moisture. After cooling in a desiccator, we recorded the spectra (400 nm to 2,500 nm at 2-nm intervals) with a NIRSystems Model 6500 Scanning Spectrophotometer (Foss NIRSystems, Laurel, MD, USA) fitted with a spinning cup module. The spectrophotometer was housed in a room at 22°C–24°C and at 55%–60% RH. We checked the accuracy of measurements at regular intervals by using a standard of known chemical composition. We converted reflectance spectra (R) to absorbance values [A=log(1/R)] and transformed them to a first-derivative spectrum to emphasize changes in slope and reduce variation resulting from differences in particle size (Osborne et al. 1993). We collected spectra from each sample in duplicate, and these were averaged once the spectral noise [the root mean square of the difference between replicates $(1/R)/10^6$)] was less than 250.

We determined foliar concentrations of N, available N, and DDM on a calibration set of 100 samples selected to represent the population of all sampled eucalypts based on a Mahalanobis distance calculation of spectral variation (WinISI II V. 1.02a; InfraSoft International, Port Matilda, PA, USA). We developed calibration equations by using a modified partial least squares regression (MPLS: Shenk and Westerhaus 1991), and a standard normal variate and detrend scatter correction (SNV detrend). We tested various combinations of Savitzy-Golay derivative-based spectral smoothing functions provided by the ISI software (Win ISI; Port Matilda, PA, USA) to remove unnecessary spectral signal components and to improve the accuracy of the prediction models. We report the best results from the application of these mathematical transformations in Table 1. The use of MPLS requires cross-validation to prevent over-fitting the model for which we used a leave-one-out (LOO) resubstitution method. The LOO cross-validation

Table 1 Results from MPLS regression with cross-validation for modeling the relationship between spectral characteristics of eucalypt foliage and foliar concentrations of nitrogen (N), available nitrogen (AvailN), and digestible dry matter (DDM)

Constituent	SNV detrend + math treatment	*N*	Mean	SD	SEC	R^2	SECV	*1-VR*
N	2841	96	1.21	0.20	0.04	0.97	0.06	0.92
AvailN	1441	78	0.37	0.31	0.06	0.96	0.10	0.90
DDM	1441	89	0.44	0.12	0.02	0.97	0.03	0.93

Math treatment refers to the Savitzy-Golay derivative based spectral smoothing and includes the derivatives and the number of data points across which the smoothing functions were calculated; SEC is standard error of calibration and SECV is standard error of cross-validation predictions; the degree of correlation between predicted and actual measured values is indicated by the R^2 and by *1-VR* for the cross-validation data. The prediction equation and validation are based on all species of *Eucalyptus* collected.

provides an estimate of model error based on data resampling (Elisseeff and Pontil 2002). We divided data into n groups and left one group out of the model in rotation so that the learning algorithm was trained multiple times on the n-1 group, while the remaining group tested the model's accuracy. We generated a standard error of cross-validation by pooling residuals from each prediction (Table 1). Finally, we validated the approach for N using 20 additional tree-leaf samples not included in the prediction model.

Chemical Assays We used freeze-dried ground material for all analyses and corrected for residual moisture by drying 1.00 g of material to constant mass at 50°C. The N content of foliage was determined on duplicate samples (250± 10 mg) using a semi-micro Kjeldahl technique calibrated with ammonium sulphate. We determined *in vitro* DDM and AvailN using the method of DeGabriel et al. (2008). This involved digesting the samples in porous bags (Ankom F57, Ankom Technology, Macedon, NY, USA), first with pepsin (24 h) and then with cellulase (48 h) to mimic digestion in a hindgut-fermenting herbivore. The purpose of the assay was to rank trees with regard to AvailN and DDM rather than trying to give precise *in vivo* values for these constituents. We discarded values if the coefficient of variation between duplicates was more than 2% (N) or 7% (AvailN and DDM) and repeated the assay.

We extracted formylated phloroglucinol compounds by sonicating single 50 mg foliage samples with a known mass (ca 4.0 g) of solvent (7% water in acetonitrile containing 0.1% trifluoroacetic acid and 0.3000 g per liter of the internal standard 2-ethylphenol) for 5 min (Wallis and Foley 2005). The resulting mixture was filtered (0.2 um) directly into an autosampler vial and then we injected 20 ul onto a Wakosil 250×4 mm GL 3C18RS (SGE Analytical: Ringwood, Australia) column maintained at 37°C with a flow rate of 0.75 ml/min on a Waters Alliance Model HPLC. We eluted the FPCs under gradient conditions with 0.1% TFA acid in water (A) and 0.1% TFA in acetonitrile (B) as follows: 40% A/60% B for 5 min, linear gradient to 90% B/10% A at 60 min, held for 10 min and returned to starting conditions over 10 min. We measured the peak response at 275 nm. We quantified the specific FPCs present by using authentic standards purified in the laboratory. The coefficient of variation between duplicate measurements is typically less than 4% (Wallis et al. 2010).

Statistical Analyses We conducted statistical analyses separately on the *Eucalyptus* (monocalypt) and *Symphyomyrtus* (symphyomyrtle) subgenera. We compared foliar concentrations of N, AvailN, and DDM among the different tree categories. These were: 1) trees in which we observed *P. volans* feeding once (feeding observation); 2) trees in which we saw an animal feeding multiple times (multiple feeding observations); 3) trees in which we observed *P. volans* in a non-feeding activity only (non-feeding observation); 4) neighboring trees with no animal observations (no observation neighbor); and 5) trees more than 20 m from any tree in which we observed an animal (no observation area). We also compared foliar concentrations of total FPCs and sideroxylonals (a class of FPC) among these tree-use categories for the symphyomyrtle species.

We combined feeding and multiple feeding trees for the following analyses due to the relatively small number of trees in each category. We assessed the distribution of data with a normal-quantile plot. Where data were normally distributed, we used ANOVAs to test the hypothesis that two population means were equal. We used a Kruskal-Wallis test when the normality assumption for ANOVAs could not be met and when our samples sizes were less than 30 but greater than 14 (Hollander and Wolfe 1999). Larger trees were less likely than smaller ones to have a suitable neighbor within a 20 m radius. Therefore, despite our effort, this resulted in a disproportionate sampling of smaller trees in the no observation categories when compared to some of the observation categories. Where significant differences were observed for several potentially confounding variables, we used logistic, binomial Generalized Linear Models (GLMs) to investigate whether a significant difference in a chemical constituent concentration remained once we controlled for other significant explanatory variables, such as the DBH.

Monocalypt trees in which we saw *P. volans* feeding repeatedly did not appear to have the same variance for N as monocalypt trees with no observations. There were sufficient multiple feeding observation trees in this monocalypt group to investigate the trend by using non-parametric statistics. First, we used an Ansari-Bradley test to determine equality of variance (Hollander and Wolfe 1999). To test whether animals avoided feeding repeatedly on trees with low concentrations of N, we determined the number of trees with no *P. volans* observations that had a lower concentration of N than any tree with multiple feeding observations. We then calculated the probability of this number or a greater number occurring by chance at either extreme of the distribution.

Results

Foliar Chemistry and Measured Tree Attributes We successfully modeled the relationship between absorbance spectra from eucalypt foliage and foliar concentrations of N, AvailN, and DDM (Table 1). The independent validation of N samples (N=20) resulted in excellent agreement between the predicted and analyzed values (R^2=0.96;

RMSE=0.042). This suggested that the samples we selected for the training algorithm by the Mahalanobis distance calculation method were representative of the spectral range of our data.

We illustrate the distributions of data for each variable according to eucalypt subgenera, monocalypt and symphyomyrtle (Fig. 2). These distributions were further segregated by tree use category (no observation area, no observation neighbor, non-feeding observation, feeding observation, and multiple feeding observations). The symphyomyrtle species (*E. viminalis* and *E. dalrympleana*) had higher concentrations of AvailN ($P<.001$, $t=22.94$, mean of the differences= 0.48% dry matter (DM)) and DDM ($P<.001$, $t=27.96$, mean of the differences=0.19 g.g^{-1} DM) than did the monocalypt

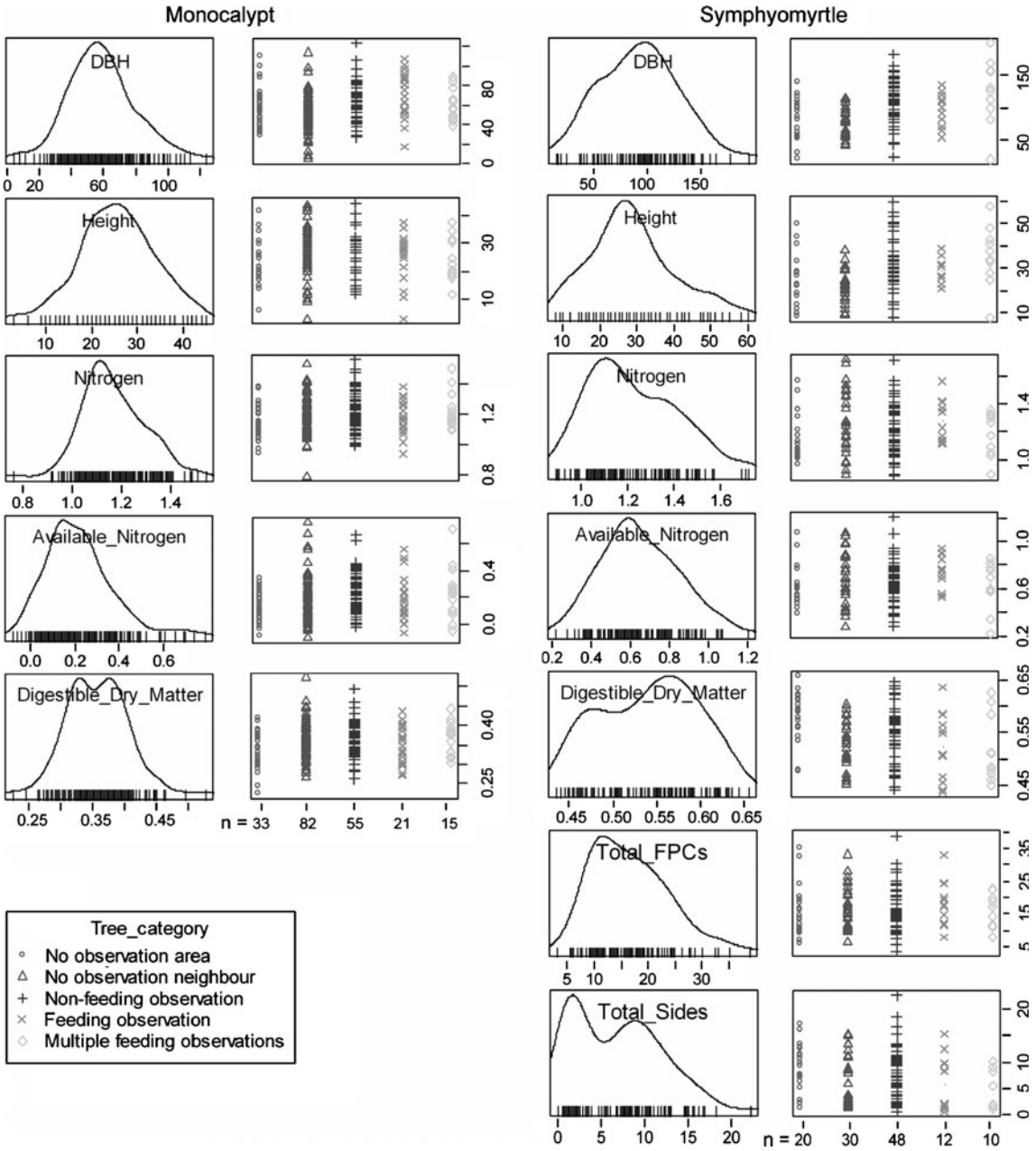


Fig. 2 Distribution of variables overall (scaled kernel density estimates) and among tree categories for monocalypts (*Eucalyptus radiata*) and symphyomyrtles (*Eucalyptus viminalis* and *Eucalyptus dalrympleana*). Variables include diameter at breast height (DBH; cm), height (m), and foliar concentrations of nitrogen (N; % dry matter (DM)), available nitrogen (AvailN; % DM), digestible dry matter (DDM; g.g^{-1} DM), total formylated phloroglucinol compounds (total FPCs; mg.g^{-1} DM), and sideroxylonals (Total Sides mg.g^{-1} DM). The sample size for each tree category is provided under the tree category distributions

species (*E. radiata*) (*P*-values from Student's *t*-test of means not included in Fig. 2).

We explored the interactions among the data by using a Pearson's correlation matrix (Table 2). The correlation between the concentrations of N and AvailN in the monocalypts was much lower ($r=0.462$, $P<0.001$) than in the symphyomyrtles ($r=0.914$, $P<0.001$). The monocalypts also showed a weak positive correlation between N and DDM ($r=0.170$, $P=0.02$). This relationship was negative in the symphyomyrtles ($r=-0.602$, $P<0.001$). Likewise, the concentration of AvailN was positively correlated with DDM in the monocalypt species ($r=0.617$, $P<0.001$) and negatively correlated in the symphyomyrtles ($r=-0.445$, $P<0.001$). When we converted tree use category to an ordinal variable, which assumed a linear relationship of increasing preference as a feeding tree from 0 to 4 (Table 2), we identified a significant correlation between tree use category and DBH for both the monocalypt ($r=0.206$, $P=0.003$) and symphyomyrtle ($r=0.376$, $P<0.001$) classes and between tree use category and tree height for symphyomyrtles ($r=0.321$, $P<0.001$). We also observed a weak but significant correlation between tree use category and AvailN ($r=0.146$, $P=0.03$) for the monocalypts and between tree use category and sideroxylonals ($r=-0.242$, $P=0.008$) in the symphyomyrtles. We identified a negative correlation for both N and AvailN with sideroxylonals ($r=0.575$, $P<0.001$ and $r=0.519$, $P<0.001$, respectively) and a positive correlation for N and AvailN with total FPCs ($r=0.669$, $P<0.001$ and $r=0.713$, $P<0.001$, respectively).

Tree Use by P. volans When comparing all symphyomyrtle trees in which we observed *P. volans* to trees with no observations, only DBH ($P<0.001$) and height ($P<0.001$) differed significantly (Table 3). Monocalypt trees in which we saw animals also had a larger DBH ($P<0.001$) than did those in which we did not observe *P. volans*. A GLM that included both DBH and AvailN (near significant in monocalypt means test, $P=0.058$) suggested that both variables were important in explaining the difference among monocalypt trees in which an animal was, and was not, observed ($P<0.001$ and $P=0.042$, respectively) (Table 4).

We separated the trees in which we did not observe *P. volans* into two categories: "no observation neighbor" and "no observation area". Trees from areas where we did not observe *P. volans* explained most of the chemical differences among categories (Table 3). Size was the only significant difference among neighboring trees in which an animal was not observed and trees in which an animal was observed. Despite our attempt to control for these differences through our sampling criteria, symphyomyrtle trees with an animal observation were significantly taller ($P<0.001$) and had a larger DBH ($P<0.001$) than did neighboring trees in which we did not see an animal. Monocalypt neighbors also had a smaller DBH ($P<0.001$) compared to the adjacent monocalypts in which we observed *P. volans*.

We found a similar difference in average height and DBH among trees sampled from areas of the forest patches where we did not observe any *P. volans* and trees in which we saw an animal (Table 3). In addition, both monocalypt

Table 2 Pearson's correlation coefficients among tree attributes

Constituent	Subgenus	AvailN	*N*	DDM	DBH	Height	Tree use category
N	Sym	**0.914**					
	Mon	**0.462**					
DDM	Sym	**−0.445**	**−0.602**				
	Mon	**0.617**	**0.170**				
DBH	Sym	−0.024	0.009	−0.138			
	Mon	−0.019	0.063	−0.020			
Height	Sym	0.068	0.054	−0.131	**0.570**		
	Mon	0.040	0.051	−0.041	**0.348**		
Tree use category	Sym	0.027	0.072	**−0.265**	**0.376**	**0.321**	
	Mon	**0.146**	0.055	0.113	**0.206**	0.028	
Total FPCs	Sym	**0.713**	**0.669**	**−0.532**	−0.008	0.124	0.009
Sideroxylonals	Sym	**−0.519**	**−0.575**	**0.667**	−0.073	−0.044	**−0.242**

Correlations among measure tree attributes for sampled eucalypt subgenera *Eucalyptus* (Mon) and *Symphyomyrtus* (Sym). Significance ($P<0.05$) is indicated in bold. Tree use category ranked from 0–4 (0 = no observation area, 1 = no observation neighbor, 2 = non-feeding observation, 3 = feeding observation, 4 = multiple feeding observations). Measured variables include diam at breast height (DBH; cm), height (m), and foliar concentrations of nitrogen (N; % dry matter (DM)), available nitrogen (AvailN; % DM), digestible dry matter (DDM; $g.g^{-1}$ DM), total formylated phloroglucinol compounds (total FPCs; $mg.g^{-1}$ DM), and sideroxylonals ($mg.g^{-1}$ DM).

Table 3 Comparison of means for measured variables among specified symphyomyrtle (**Sym**) and monocalypt (**Mon**) tree categories

	All non-observation trees vs All observation trees			No observation neighbour vs All observation trees			No observation area vs All observation trees			No observation neighbour vs Feeding observations			No observation area vs Feeding observations			Non-feeding observation vs Feeding observations		
	Mean	Test statistic	*P*-value	Mean	Test statistic	*P*-value	Mean	Test statistic	*P*-value	Mean	Test statistic	*P*-value	Mean	Test statistic	*P*-value	Mean	Test statistic	*P*-value
Sym Variable																		
DBH	68.9 101.3	F=28.80	**<0.001**	65.5 101.3	F=24.63	**<0.001**	73.7 101.3	χ^2 =9.20	**0.002**	65.5 100.2	χ^2=11.4	**<0.001**	73.7 100.2	χ^2 =5.03	**0.025**	101.8 100.2	χ^2 =1.05	0.306
Height	26 34	F=20.16	**<0.001**	24 34	F=18.38	**<0.001**	27 34	χ^2 =6.70	**0.010**	24 34	χ^2 =11.87	**<0.001**	27 34	χ^2 =3.72	0.054	35 34	χ^2=0.01	0.929
N	1.22 1.22	F=0.04	0.834	1.27 1.23	F=1.00	0.320	1.14 1.22	χ^2 =5.18	**0.023**	1.27 1.23	χ^2 =0.21	0.650	1.14 1.23	χ^2 =5.37	**0.021**	1.22 1.23	χ^2 =0.30	0.582
DDM	0.55 0.54	χ^2 =1.05	0.311	0.53 0.54	F=1.14	0.288	0.58 0.53	χ^2 =9.83	**0.002**	0.53 0.52	χ^2=0.12	0.725	0.58 0.52	χ^2 =9.29	**0.002**	0.55 0.52	χ^2 =2.31	0.129
Total Sides	7.35 6.27	χ^2 =1.34	0.250	5.67 6.27	χ^2 =0.01	0.933	9.37 6.24	χ^2 =6.22	**0.013**	5.97 4.63	χ^2=2.80	0.094	9.37 4.64	χ^2 =9.13	**0.003**	7.02 4.64	χ^2 =5.27	**0.022**
Mon Variable																		
DBH	53.4 65.9	F=20.97	**<0.001**	52.5 65.9	F=20.61	**<0.001**	55.6 65.9	F=6.48	**0.012**	52.5 64.8	F=9.67	**0.002**	55.6 64.8	F=3.35	0.072	66.6 64.8	χ^2 =0.27	0.605
Height	25 26	F=0.51	0.475	26 26	F=0.05	0.817	24 26	F=1.79	0.184	26 25	F=0.52	0.473	24 25	F=0.11	0.740	27 25	χ^2 =1.48	0.224
N	1.19 1.21	F=1.46	0.229	1.20 1.21	F=0.11	0.736	1.15 1.21	χ^2 =4.24	**0.040**	1.20 1.20	F=0.01	0.939	1.15 1.20	F=3.63	0.061	1.21 1.20	χ^2 =0.01	0.942
AvailN	0.18 0.22	χ^2 =3.60	0.058	0.19 0.22	χ^2 =1.16	0.281	0.13 0.22	χ^2 =6.53	**0.011**	.19 .21	F=0.23	0.635	0.13 0.21	F=4.62	**0.035**	0.22 0.20	χ^2 =0.44	0.506
DDM	0.35 0.36	F=1.69	0.195	0.36 0.36	F=0.31	0.577	0.34 0.36	F=4.54	**0.035**	.36 .36	F=7e-04	0.979	0.34 0.36	F=2.07	0.155	0.36 0.36	χ^2 =0.29	0.592

Significance ($P \leq 0.05$) is indicated in bold. Measured variables include diam at breast height (DBH; cm), height (m), and foliar concentrations of nitrogen (N; % dry matter (DM)), available nitrogen (AvailN; % DM), digestible dry matter (DDM; $g.g^{-1}$ DM), total formylated phloroglucinol compounds ($mg.g^{-1}$ DM), and sideroxylonals (Total Sides; $mg.g^{-1}$ DM). Variables that were insignificant for all monocalypt or symphyomyrtle tree category comparisons were excluded from the table

Table 4 Generalized linear models of significant explanatory variables among specified monocalypt and symphyomyrtle tree categories

glm(formula) = Tree category ~ DBH + Available N, family = binomial(logit), data = *monocalypt*

All non-observation trees vs All observation trees

Coefficients:	Estimate	Std. Error	Z value	Pr(>|z|)
(Intercept)	−2.687	0.562	−4.779	**<0.001**
DBH	0.003	0.001	4.224	**<0.001**
AvailN	2.017	0.991	2.035	**0.042**

glm(formula) = Tree category ~ DBH + N, family = binomial (logit), data = *monocalypt*

No observation area vs All observation trees

(Intercept)	−5.262	2.391	−2.201	**0.028**
DBH	0.003	0.001	2.333	**0.020**
N	3.922	1.944	2.017	**0.044**

glm(formula) = Tree category ~ DBH + Height + Total Sides, family = binomial(logit), data = *symphyomyrtle*

No observation area vs Feeding observation

(Intercept)	−0.621	1.467	−0.423	0.672
DBH	0.001	0.001	0.830	0.407
Height	0.033	0.038	0.873	0.383
Total Sides	−0.166	0.077	−2.161	**0.031**

glm(formula) = Tree category ~ DBH + Height + Total Sides, family = binomial(logit), data = *symphyomyrtle*

No observation area vs All observation trees

Coefficients:	Estimate	Std. Error	Z value	Pr(>|z|)
(Intercept)	−0.355	0.976	−0.364	0.716
DBH	0.002	0.001	1.651	0.099
Height	0.035	0.031	1.130	0.258
Total Sides	−0.119	0.058	−2.069	**0.039**

glm(formula) = Tree category ~ DBH + Digestible Dry Matter, family = binomial(logit), data = *monocalypt*

No observation area vs All observation trees

(Intercept)	−3.990	1.832	−2.178	**0.029**
DBH	0.003	0.001	2.351	**0.019**
DDM	9.488	4.810	1.972	**0.049**

glm(formula) = Tree category ~ DBH + Height + Digestible Dry Matter, family = binomial(logit), data = *symphyomyrtle*

No observation area vs Feeding observation

(Intercept)	7.369e+00	4.197e+00	1.756	0.079
DBH	6.469e−04	1.257e−03	0.515	0.607
Height	4.085e−02	3.930e−02	1.040	0.299
DDM	−1.635e+01	6.810e+00	−2.400	**0.016**

glm(formula) = Tree category ~ Height + DBH + Digestible Dry Matter, family = binomial(logit), data = *symphyomyrtle*

No observation area vs All observation trees

Coefficients:	Estimate	Std. Error	Z value	Pr(>|z|)
(Intercept)	7.054e+00	3.533e+00	1.996	**0.046**
DBH	1.608e−03	9.795e−04	1.642	0.101
Height	2.585e−02	3.177e−02	0.814	0.416
DDM	−1.424e+01	5.896e+00	−2.415	**0.016**

glm(formula) = Tree category ~ DBH + Available N, family = binomial(logit), data = *monocalypt*

No observation area vs All observation trees

(Intercept)	−1.590	0.789	−2.015	**0.044**
DBH	0.003	0.001	2.513	**0.012**
AvailN	4.561	1.702	2.680	**0.007**

glm(formula) = Tree category ~ DBH + Available N, family = binomial(logit), data = *monocalypt*

No observation area vs Feeding observation

(Intercept)	−2.195	0.925	−2.372	**0.018**
DBH	0.003	0.001	1.995	**0.046**
AvailN	4.139	1.939	2.134	**0.033**

Significance ($P \leq 0.05$) is indicated in bold. Variables include diam at breast height (DBH; cm), height (m), and foliar concentrations of nitrogen (N; % dry matter (DM)), available nitrogen (AvailN; % DM), digestible dry matter (DDM; $g.g^{-1}$ DM), and sideroxylonals (Total Sides; $mg.g^{-1}$ DM)

and symphyomyrtle trees from these two categories differed chemically. The GLMs that controlled for potentially confounding size variables showed that sideroxylonals (P=0.039) and DDM (P=0.016) explained a significant amount of the variation among symphyomyrtle tree populations from areas where *P. volans* was and was not observed (Table 4). Likewise, N (P=0.044), available N (P=0.007), and DDM (P=0.049) explained differences in tree use between these two tree categories for monocalypt trees.

Symphyomyrtle trees in which we observed *P. volans* feeding were taller (P<0.001) and had a larger DBH (P< 0.001) than neighboring trees with no animal observations (Table 3). Monocalypt trees from the same categories differed only in mean DBH (P=0.002). We found this same trend in height (P=0.054) and DBH (P=0.025) among symphyomyrtle trees sampled from areas where we did not observe *P. volans*, and among trees in which we observed *P. volans* feeding. Monocalypt trees from these categories had no significant difference in DBH (P=0.072). Allowing for tree size, GLMs showed that symphyomyrtle trees with feeding observations had lower concentrations of sideroxylonals (P=0.031) and DDM (P=0.016) than did trees from areas where *P. volans* was not observed (Table 4). Similarly, GLMs showed that monocalypt trees in which we observed *P. volans* feeding were likely to have more AvailN (P=0.033) and have a larger DBH (P=0.046) than trees sampled from areas where we did not see any animals. A monocalypt GLM that included N and DBH showed no significant values for either attribute (P=0.096 and P=0.071, respectively).

Symphyomyrtle trees in which we observed an animal feeding had lower concentrations of sideroxylonals (P= 0.022) than did trees in which we observed *P. volans* in a non-feeding activity only (Table 3). Monocalypt trees from these categories did not differ chemically.

Monocalypt trees in which we repeatedly observed *P. volans* feeding had less variable N concentrations (P= 0.041) than did trees in which we did not observe the animal. It appears that *P. volans* avoided feeding repeatedly from trees with N concentrations that reflected the lower range of measured N values—as found in the distribution of foliar N among all no observation trees (P=0.048) and no observation area trees (P=0.017).

Discussion

Tree Use and Foliar Chemistry The eucalypt trees in which we observed *P. volans* differed chemically from trees of the same species in nearby areas with no animal observations. In addition, symphyomyrtle trees in which animals fed differed chemically from the symphyomyrtle trees in which we observed *P. volans* engaged in a non-feeding activity only. Our findings refute the null hypothesis that there are no chemical differences among trees in which we observed *P. volans* and those in which we did not. The study demonstrates that intraspecific variations in sideroxylonals and DDM concentrations in the foliage of *E. viminalis* and *E. dalrympleana* and of N, AvailN, and DDM in *E. radiata*, play a role in tree use and hence habitat suitability for *P. volans*.

Subgenera Dependent Responses of P. volans to Foliar Chemistry The foliage of trees from the two major eucalypt subgenera pose different problems for foraging animals. The lower average concentrations of AvailN in the monocalypt, *E. radiata,* compared with the symphyomyrtles, *E. viminalis* and *E. dalrympleana,* may explain why this constituent had a significant positive relationship with *P. volans* tree use in the monocalypt species that was not seen in the other subgenus. Our data also suggest that *P. volans* may not forage repeatedly in *E. radiata* trees with concentration of foliar N below a particular threshold (around 1% dry matter). This finding is consistent with other research indicating that foliar N may be a limiting environmental factor for *P. volans* (Foley and Hume 1987; Kavanagh and Lambert 1990). In contrast, the concentration of sideroxylonals was more important than was any measure of N for influencing the use of *E. viminalis* and *E. dalrympleana*.

The concentration of DDM played opposing roles in explaining differences among tree categories for monocalypts and symphyomyrtles. This measure of forage digestibility is governed largely by a negative relation to concentrations of foliar lignin and phenols (e.g., tannins) (Choo et al. 1981). Presumably, the indigestible dry matter of monocalypts contains much of the foliar N bound by tannins and as a result, DDM had a stronger positive correlation to foliar concentrations of AvailN than N. This supports the pattern of tree use by *P. volans* when foraging in *E. radiata*; they should seek trees whose foliage contains lower concentrations of tannins and thus higher concentrations of available nitrogen.

In contrast, we observed a negative relationship between DDM and concentrations of both N and AvailN in the symphyomyrtle species. We also found a positive correlation between the concentrations of DDM and sideroxylonals. These correlations create a nutritional dilemma for animals eating those leaves because the most digestible components of the foliage contain the most sideroxylonal but the least N and AvailN, which may be bound to other non-digestible lignin compounds (Cork and Foley 1991; Dyckmans et al. 2002). We would not expect this strong negative relationship between DDM and N in immature leaves, which have a higher ratio of N to ligno-cellulose than do mature leaves (Kavanagh and Lambert 1990; Cork

and Foley 1991). The age variability of this ratio is greater for *E. viminalis* than for other species like *E. radiata* and, not surprisingly, some field studies have reported that *P. volans* will forage preferentially on young leaves, particularly from *E. viminalis* (Kavanagh and Lambert 1990). We propose that the opposing roles that DDM played in explaining *P. volans* tree use in the two subgenera results from the relationship between DDM and these other foliage constituents.

Relevance of Feeding Observations and Spatial Considerations for Determining Relationship between Foliar Chemistry and Tree Use Specific observations of feeding strengthened the negative relationship between the choice of feeding tree and the sideroxylonal concentration of foliage for *E. viminalis* and *E. dalrympleana*. Feeding-specific observations also were useful in identifying significant differences in the variance of N concentrations among tree categories for the monocalypt species. In most instances, however, feeding observations were not required to identify chemical differences among tree categories. This concurs with Moore and Foley (2005), who showed that we could learn much about the chemistry of trees that were preferred by *P. cinereus* from animal location data alone.

We found that the proximity of sampled trees to any tree in which *P. volans* was observed was important to identify a relationship between foliar chemistry and tree use. Most of the differences among trees in which we saw an animal and those in which we did not were apparent only when the "no observation trees" were more than 20 m from any tree with an observation. This suggests a spatial genetic effect similar to that shown for the concentration of PSMs in the foliage of *E. melliodora*, whereby individual trees within 20 m of each other tended to be more closely related and thus more similar chemically than more distant trees (Andrew et al. 2007).

Differences in the physical characteristics of the monocalypt and symphyomyrtle species that we examined may affect spatial distributions and influence their use by *P. volans*. Both *E. viminalis* and *E. dalrympleana* grow taller and wider leaving more space between mature trees than typically occurs for *E. radiata*. Consequently, there were fewer appropriate neighbor trees for the symphyomyrtles than for the monocalypts. In addition, symphyomyrtle trees in which we saw *P. volans* in activities other than feeding often were more than 20 m from any feeding-observation tree. These differing spatial relationships could explain why non-feeding and feeding tree categories differed only for the symphyomyrtles.

Foraging Niche of P. volans Herbivores that forage primarily from a single plant genus or family, such as *P. volans*, are often labeled as "specialists" (Lindenmayer 1997; Shipley et al. 2009). However, chemical and physical attributes that can vary substantially among plants within the same species and that may occur across plant taxa generally show a stronger association to herbivore dietary specialization than does plant taxonomy (Shipley et al. 2009). In hypothesizing about the foraging niche of marsupial eucalypt folivores, Moore et al. (2004) placed *P. volans* with *P. peregrinus*, a monocalypt and tannin specialist that also consumes non-eucalypt foliage. *Pseudocheirus peregrinus* is the smallest marsupial folivore and uses caecotrophy and a specialized caecum to free N from tannins in the gut (McArthur and Sanson 1991; Marsh et al. 2003a). Consequently, laboratory studies have found only a weak relationship between feeding choices and concentrations of tannins for this species (Marsh et al. 2003b). *Petauroides volans* also feeds on tannin-rich monocalypt species, and both species can survive on a diet of monocalypt foliage in captivity (Chilcott and Hume 1984; Foley and Hume 1987). We found, however, that the activity of tannins, as indicated by the concentration of AvailN in the leaves, strongly influenced its tree use. This suggests that *P. volans* is more sensitive to tannins and their influence on N availability than is *P. peregrinus*. If *P. volans* is not as efficient at liberating tannin bound N, then it may need to feed on the more AvailN-rich symphyomyrtle foliage, particularly when N requirements increase during pregnancy or lactation or in sites where low soil N is reflected in low foliar N. It is important to recognize that this hypothesis is based on the comparison of an *in vivo* laboratory study with a field study that relied on *in vitro* methodology and requires further laboratory investigations to verify.

Petauroides volans, like other eucalypt folivores, can consume foliage containing sideroxylonals, although it showed a strong preference for foliage with lower concentrations of these chemicals. In addition, both *E. viminalis* and *E. dalrympleana* contain low concentrations of sideroxylonals, compared with some symphyomyrtle species that other arboreal folivores eat that contain high levels of sideroxylonals but no other FPCs (e.g., *E. melliodora* and *E. polyanthemos*; Wallis et al. 2002). We would not expect *P. cinereus* or *T. vulpecula* to feed selectively when faced with the concentrations of sideroxylonals that influenced tree choice in *P. volans* (Lawler et al. 1998a; Wallis et al. 2002). Unlike *P. cinereus*, *P. volans* also showed a stronger response to the concentration of sideroxylonals than to total FPCs (Moore et al. 2005). Laboratory feeding studies are necessary to determine whether *P. volans* is less sensitive to other FPC derivatives, such as macrocarpals. In addition, research is necessary to determine how concentrations of PSMs and nutrients influence feeding behavior on immature leaves, since we conducted our analyses on mature leaf samples only. We do not know whether *P. volans*, like *P. cinereus* and *T. vulpecula*, can persist on a diet of

symphyomyrtle foliage alone (Moore et al. 2004); however, it appears that *P. volans* is very susceptible to the physiological effects of sideroxylonals while also lacking the adaptations of *P. peregrinus* to cope with high levels of tannins.

Studies of captive animals may show that the strategy of *P. volans* is intermediate between that of *P. peregrinus*, which specializes on foliage containing large concentrations of tannins, and that of *P. cinereus*, which specializes on foliage with significant concentrations of FPCs. In terms of PSM specialization, we propose that *P. volans* is more of a generalist than *P. peregrinus* or *P. cinereus*. A test of the detoxification limitation hypothesis (reviewed by Marsh et al. 2006) should reveal that *P. peregrinus* and *P. cinereus* have a higher rate of foliage consumption and detoxification of PSMs from their preferred subgenus than does *P. volans*. However, *P. volans* should be able to eat more when presented with foliage from preferred monocalypt and symphyomyrtle species simultaneously, each requiring different PSM detoxification pathways. In other words, *P. volans* will eat foliage from the symphyomyrtle species until the physiological effects of the FPCs force it to stop. Because monocalypts do not contain FPCs, *P. volans* can consume the foliage of *E. radiata* while detoxification processes remove the FPCs. It then can revert to eating symphyomyrtle foliage before ingesting debilitating amounts of tannins in the monocalypts (Marsh et al. 2006).

Petauroides volans may use this strategy to maintain its nutrient and energy requirements despite its small body size and poor-quality diet. Observations of free-living *P. volans* in our study area suggest that it may divide its feeding time between monocalypt and symphyomyrtle tree species (Cunningham et al. 2004). Energy and detoxification requirements, coupled with the general paucity of nutrients in eucalypt foliage, may have favored the development of a more energy efficient means of moving through the forest, namely gliding. This adaptation also would enable *P. volans* to take advantage of favorable variations in foliage chemistry as a result of leaf age and/or distribution of genetic variability in both major eucalypt subgenera at a minimal energy cost compared to other forms of movement (Jackson 2000).

Conservation Implications Factors that confine an animal's use of the landscape, such as a highly selective diet, make them particularly susceptible to landscape change. *Petauroides volans* and other herbivores that persist on nutritionally-poor foliage or foliage that contains high levels of certain PSMs may be vulnerable to environmental changes that could further reduce the nutritional quality or increase the toxicity of their forage (Lawler et al. 1997; Zvereva and Kozlov 2006). Higher levels of atmospheric CO_2 have been shown to increase the production of tannins and reduce the concentration of N in plant tissues (Lawler et al. 1997; Gleadow et al. 1998; Mansfield et al. 1999). Thus, some plants grown under elevated CO_2 have reduced herbivore damage (Knepp et al. 2005). The potential effects of climate change on nutrient availability and plant toxicity need study (Lindroth 2010; Pinto et al. 2010).

We recommend that land managers give preference to conserving tracts of eucalypt forest where marsupial folivores occur. Areas where animals are not sighted may not have the same capacity to support them for several reasons, including inadequate forage quality. The ability to accurately measure variation in plant nutrients and PSMs on a landscape scale could become an important conservation tool. Large-scale mapping of foliage chemistry could help to identify areas that are suitable or unsuitable habitat for certain animals and to monitor changes to the chemical quality of landscapes that could influence their ability to support some species.

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Male *Phyllotreta striolata* (F.) Produce an Aggregation Pheromone: Identification of Male-specific compounds and Interaction with Host Plant Volatiles

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Abstract The chrysomelid beetle *Phyllotreta striolata* is an important pest of Brassicaceae in Southeast Asia and North America. Here, we identified the aggregation pheromone of a population of *P. striolata* from Taiwan, and host plant volatiles that interact with the pheromone. Volatiles emitted by feeding male *P. striolata* attracted males and females in the field. Headspace volatile analyses revealed that six sesquiterpenes were emitted specifically by feeding males. Only one of these, however, elicited an electrophysiological response from antennae of both sexes. A number of host plant volatiles, e.g., 1-hexanol, (*Z*)-3-hexen-1-ol, and the glucosinolate hydrolysis products allyl isothiocyanate (AITC), 3-butenyl isothiocyanate, and 4-pentenyl isothiocyanate also elicited clear responses from the antenna. The active male-specific compound was identified as (+)-(6*R*,7*S*)-himachala-9,11-diene by chiral stationary phase gas-chromatography with coupled mass spectrometry, and by comparison with reference samples from *Abies nordmanniana*, which is known to produce the corresponding enantiomer. The pheromone compound was synthesized starting from (–)-α-himachalene isolated from *Cedrus atlantica*. Under field conditions, the activity of the synthetic pheromone required concomitant presence of the host plant volatile allyl isothiocyanate. However, both synthetic (+)-(6*R*,7*S*)-himachala-9,11-diene alone and in combination with AITC were attractive in a two-choice laboratory assay devoid of other natural olfactory stimuli. We hypothesize that *P. striolata* adults respond to the pheromone only if

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specific host volatiles are present. In the same laboratory set up, more beetles were attracted by feeding males than by the synthetic stimuli. Thus, further research will be necessary to reveal the components of a more complex blend of host or male-produced semiochemicals that might enhance trap attractiveness in the field.

Key Words *Brassica* · Chrysomelidae · Coleoptera · *Phyllotreta striolata* · Aggregation pheromone · Host plant volatiles · Gas chromatography-mass spectrometry (GC-MS) · Glucosinolate hydrolysis products · Allyl isothiocyanate · (+)-(6*R*,7*S*)-himachala-9,11-diene

Introduction

Flea beetles (Alticinae) of the genus *Phyllotreta* (Chevrolat) (Coleoptera: Chrysomelidae) are specialized herbivores on the Brassicaceae and related plant families. *Phyllotreta striolata* (Fabricius) is a pest of economically important *Brassica* crops (Lamb, 1989) and widely distributed in North America, Europe, and Asia. In Southeast Asia, it is the dominant *Phyllotreta* species (Parker et al., 1995; Mohamedsaid, 2004) with up to eleven generations per year (Chen et al., 1991). The adults feed mainly on host plant leaves, and females oviposit at the plant–soil interface. The larvae feed on root hairs of host plants, and pupate in the soil (Feeny et al., 1970; Burgess, 1977).

Glucosinolates are characteristic secondary metabolites in the Brassicaceae (Fahey et al., 2001), and the role of the glucosinolate–myrosinase system in host plant location and acceptance of *Phyllotreta* sp. has been studied intensively in the past (Nielsen, 1988; Mitchell, 1994; Nielsen et al., 2001). Upon feeding, glucosinolates are hydrolyzed by the plant enzyme myrosinase, producing a blend of volatile and non-volatile hydrolysis products that represent potential host-finding cues for specialists such as *P. striolata* (Renwick, 2002). Field trapping experiments have shown that only a few volatile glucosinolate hydrolysis products are attractive for adult flea beetles (Görnitz, 1956; Feeny et al., 1970; Pivnick et al., 1992; Csonka et al., 2007). The hydrolysis product of allyl glucosinolate, allyl isothiocyanate (AITC), is a plant-derived attractant for several *Phyllotreta* species in the field, including *P. striolata* (Pivnick et al., 1992; Tóth et al., 2007). However, in order to catch high numbers of flea beetles, an evaporation rate of 4 mg AITC per day per trap, corresponding to the release from 2,000 freshly damaged plants or 270,000 intact plants of *Brassica juncea* (L.) in the flower bud stage, is necessary (Pivnick and Jarvis, 1991; Pivnick et al., 1992). It is, therefore, unlikely that AITC is a key stimulus used for host plant location by *Phyllotreta* sp. on its own. Recently, several host plant volatiles, e.g., 1-hexanol, 1-pentanol, and (*Z*)-3-hexen-1-ol, were shown to be more attractive than AITC for the congeneric species *Phyllotreta cruciferae* (Goeze) in olfactometer bioassays (Gruber et al., 2009).

Male-produced aggregation pheromones have been described for several chrysomelid subfamilies, e.g., Alticinae, Chrysomelinae, Criocerinae, and Galerucinae. In most cases, however, the synthetic pheromone compounds alone are only weakly attractive (Cossé et al., 2002; Dickens et al., 2002; Rao et al., 2003; Soroka et al., 2005; Tóth et al., 2005; Bartelt et al., 2006; Zilkowski et al., 2006). Peng and Weiss (1992) first provided evidence for a male-produced aggregation pheromone in *P. cruciferae,* and subsequently six male-specific sesquiterpenes were identified, four of which have been synthesized (Bartelt et al., 2001, 2003; Muto et al., 2004). In field trapping experiments, the synthetic compounds alone were not or only slightly attractive. The sesquiterpene (+)-(6*R*,7*S*)-himachala-9,11-diene was identified as the only behaviorally active compound of *P. cruciferae* (Tóth et al., 2005).

Host plant volatiles are known to affect the response to pheromones of phytophagous insects (Landolt and Phillips, 1997; Reddy and Guerrero, 2004; Seybold et al., 2006). For example, the leaf beetle, *Diorhabda elongata* (Brullé), is attracted to green leaf volatiles emitted from its host plant salt cedar, *Tamarix* spp., and these compounds interact synergistically with the male-produced aggregation pheromones of this species (Cossé et al., 2006). Similarly, Colorado potato beetles, *Leptinotarsa decemlineata* (Say), in y-tube experiments prefer a combination of the aggregation pheromone with host plant volatiles over the aggregation pheromone alone (Dickens, 2006). Also, in *P. cruciferae*, the aggregation pheromone enhances the attractiveness of the host plant volatile AITC synergistically (Soroka et al., 2005; Tóth et al., 2005).

The objective of this study was to identify the aggregation pheromone of the Taiwanese population of *P. striolata*, and, by using both plants and synthetic compounds, to study how host plant volatiles interact with the pheromone.

Methods and Materials

Insects Adult *Phyllotreta striolata* were collected from fields with crucifer crops at AVRDC-The World Vegetable Center in Shanhua, Taiwan, and maintained in cages with potted *Brassica juncea* cv. Bau-Sin plants. Eggs were collected by washing the leaves. They were subsequently transferred to pots with *Brassica rapa* var. *chinensis* cv. Ching-Chiang plants. After 2 wk, plants were cut, and pots were covered with fine mesh until adults emerged. Adults used in laboratory experiments were shipped to the Max Planck

Institute for Chemical Ecology, Jena, Germany,[1] where they were maintained on savoy cabbage, *B. oleracea* var. *sabauda* in an environmental chamber at 25°C, 75% humidity, and L12:D12 until use. The sex of *P. striolata* adults was determined based on the sexual dimorphism of the antennae. The fifth antennomer is enlarged in males, whereas it is about the same diameter as the other antennal segments in females (Heikertinger, 1941).

Plants Seeds of *B. juncea* cv. Bau Sin and *B. rapa* var. *chinensis* cv. Ching-Chiang were purchased from Known-You Seed Co. LTD, Taiwan. Savoy cabbage, *B. oleracea* var. *sabauda*, was purchased at a local grocery store. *Abies nordmanniana* twigs were obtained from the botanical garden in Jena, Germany.

Volatile Collections Headspace collections were carried out in a closed loop system operated by a rotary vane vacuum and pressure pump (model G12/01 EB, Gardner Denver, Inc., Puchheim, Germany), equipped with stainless steel adapters (IQSG-M54 Jenpneumatik, Jena, Germany), creating an airflow of 0.5 l/min though the system. All tubings was made of Teflon (OD 4 mm). Samples were placed in a silanized 50 ml flask closed with a custom made polyether ether ketone (PEEK) stopper. The incoming air stream was purified on an activated charcoal filter before entering the flask though a stainless steel exhaust silencer (SSD50, Jenpneumatik, Jena, Germany). Volatiles were trapped on an activated charcoal filter (1.5 mg CLSA filter, Gränicher & Quartero, Daumazan sur Arize, France) attached to the stopper with a custom made PEEK adapter. Volatiles emitted from 30 male or female *P. striolata* feeding on one cut *B. juncea* cv. Bau Sin leaf were collected for 12 or 24 hr in the laboratory. Beetles and foliage were changed for each volatile collection. Headspace volatiles from cut *A. nordmanniana* needles were collected for 24 hr. Filters were eluted with 30 µl hexane containing 10 ng/µl bromodecane (Sigma-Aldrich) as an internal standard. Between volatile collections all parts were cleaned. Glassware, teflon, and PEEK were heated at 230°C for 2 hr; adapters with plastic and rubber parts were heated at 90°C for 2 hr in a drying oven.

Gas Chromatography-Mass Spectrometry (GC-MS) Volatile samples were analyzed by coupled gas chromatography-mass spectrometry by using an Agilent 6890 N gas chromatograph (GC) equipped with an HP-5MS capillary column (30 m×0.25 mm ID with 0.25 µm film thickness) and coupled to a quadrupole mass spectrometer Agilent 5975B. The carrier gas was helium at a constant flow rate (1.1 ml/min). Injection temperature was 250°C. The oven program started at 40°C for 3 min and increased at 10°C/min to 280°C. One µl of each sample was injected in splitless mode. MS conditions were electron impact mode (70 eV), and scan mode 33–350 amu. Bartelt et al. (2001) reported that several flea beetle compounds decompose at high injector temperatures (250°C). Thus several samples also were analyzed with the injector temperature set to 200°C without finding any impact of the lowered injector temperature on the composition of the samples. Compounds were identified by comparison of mass spectra to the NIST (National Institute of Standards, USA) mass spectral library and confirmed by using authentic reference substances purchased from commercial sources, unless otherwise stated. The sesquiterpene content was calculated as TIC peak area relative to the internal standard bromodecane. The configuration of chiral compounds was ascertained via GC with a chiral stationary phase (Cyclosil-B column, 30 m×0.25 mm ID with 0.25 µm film thickness, Agilent) and comparison of mass spectra and retention times to samples of known chemical composition. The oven program was 60°C for 1 min and increased at 1°C/min (chiral analysis of compound A) or 3°C/min (compounds B and D) to 160°C and at 30°C/min to 200°C held for 2 min. The carrier gas was helium at a constant flow rate (1.1 ml/min). MS parameters were as stated above.

Electrophysiology Physiologically active volatiles from male *P. striolata* feeding on host plant leaves were identified by gas chromatography coupled with electroantennographic detection (GC-EAD). Volatile collections were separated on an Agilent 6890 N GC equipped with an HP-5MS capillary column (30 m×0.32 mm ID with 0.25 µm film thickness, Agilent). The carrier gas was helium with a constant flow rate (2 ml/min). The oven program started at 40°C for 3 min and increased at 20°C/min to 280°C. The GC effluent was split 1:1 at a Graphpack 3D/2 cross piece (Gerstel, Mülheim, Germany) to a flame ionization detector (FID) and an electroantennographic detector with He (40 kPa) added as make up gas. GC effluents were carried at 280°C though a flexible transfer line (ODP3, Gerstel) to the electroantennographic detector. A permanent flow of 0.5 l/min clean moistened air was added to the GC effluents within the ODP head. A PEEK outlet (ID 7 mm) carried this air stream to the antennal preparation. Pasteur pipettes loaded with 100 ng AITC in 10 µl hexane or 10 µl pure hexane on filter paper served to deliver reference stimulus pulses before and after each GC-EAD run though a hole in the PEEK outlet (ID 2 mm). Pulses had 0.5 s duration at a flow rate of 0.8 l/min. Airflow and pulses were controlled by a CS-55 stimulus controller (Syntech, Hinterzarten, Germany). Antennal responses were recorded from beetles restrained in a cut pipette tip with one antenna immobilized. Connections to

[1] Import authorization obtained under Dir 2008/61/EC

the recording unit were established with the antennal tip inserted into an Ag/AgCl glass capillary electrode filled with Ringer solution (Kaissling, 1971). The reference electrode was inserted into an eye. EAD responses were amplified 10× by using a Universal AC/DC Probe connected to an IDAC 4 signal converter (Syntech, Hinterzarten, Germany). GC-EAD runs were recorded on a standard PC with Syntech GC-EAD Software. Volatile collections from feeding male *P. striolata* were analyzed by using antennae from at least five females and males, respectively.

Retention time indices (Van Den Dool and Kratz, 1963) were calculated based on injections of C7-C26 alkanes to identify GC peaks across instrumental conditions. Physiological responses of putatively identified compounds were ascertained by using authentic reference compounds as indicated.

Synthesis of (+)-(6R,7S)-himachala-9,11-diene The putative aggregation pheromone was prepared in the steps from (–)-α-himachalene (**1**) (Fig. 1). In the first step, the exocyclic double bond of (–)-α-himachalene was catalytically hydrogenated to yield a mixture of diastereomeric products. In the second step, the remaining double bond was brominated, and finally, elimination of two molecules of hydrogen bromide yielded the target compound (**4**) and byproducts. Nuclear magnetic resonance (NMR) spectra

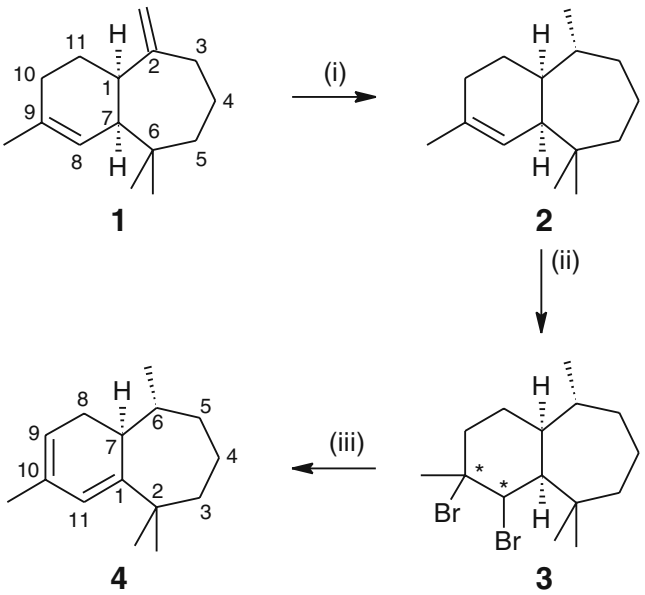


Fig. 1 Preparation of (+)-(6*R*,7*S*)-himachala-9,11-diene (**4**) from (–)-α-himachalene (**1**) (1*R*,7*S*). Conditions: (i) hydrogen (g), platinum(iv) oxide, ethanol, room temperature, only the (1*S*,2*R*)-isomer is shown. (ii) bromine, chloroform, 0–5°C, 2 h. Only the (1*S*,2*R*)-isomers are shown. The configuration at C-8 and C-9 is not known. (iii) DBU, THF, room temperature. Only the desired stereoisomer (6*R*,7*S*) is shown. IUPAC numbering is used

were recorded on a Bruker Avance DRX 500 NMR spectrometer. The spectra are referenced to the residual solvent signal. Thin-layer chromatography (TLC) was carried out on 4×8 cm silica-coated glass plates. Compounds were detected by cerium ammonium nitrate staining. Mass spectra were measured on a ThermoQuest Trace GC 2000 Series–ThermoQuest MS dyad (CE Instruments, Austin, TX, USA) using a ZB-5 column (15 m with 10 m pre-column×0.25 mm ID with 0.25 μm film thickness, Phenomenex, Aschaffenburg, Germany). The carrier gas was helium with a constant flow rate (1.5 ml/min). The oven program started at 50°C for 1 min and increased at 15°C/min to 300°C. Injection temperature was 280°C. MS condition was electron impact mode (70 eV). High resolution mass spectra were measured on a MasSpec (Micromass, UK) coupled with an HP 6890 GC (Agilent) using a ZB-5MS column (30 m×0.25 mm ID with 0.25 μm film thickness, Phenomenex). The carrier gas was helium at a constant flow rate (1 ml/min). Injection temperature was 220°C. MS condition was electron impact mode (70 eV).

The starting compound (–)-α-himachalene and the reference compound (+)-β-himachalene were distilled from *Cedrus atlantica* essential oil under reduced pressure (2.5 mbar) though a 170 cm column filled with Sulzer DX packing (Sulzer Chemtech AG, Switzerland). Both (–)-α-himachalene and (+)-β-himachalene were obtained with a purity each of 97.5%. Relative stereochemistry of both sesquiterpenes was ascertained with NMR (Joseph and Dev, 1968; Sano and Mori, 1999).

(–)-α-himachalene (αD20=–181, neat, 1 dm):

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.96 (s, 3 H), 1.00 (s, 3 H), 1.11–1.18 (m, 1 H), 1.24–1.37 (m, 1 H), 1.54–1.65 (m, 2 H), 1.67 (s, 3 H), 1.72–1.95 (m, 4 H), 2.04 (t, J=12.3 Hz, 1 H), 2.10–2.21 (m, 2 H), 2.80–2.84 (m, 1 H), 4.72 (s, 1 H), 4.76 (s, 1 H), 5.48 (s, 1 H).

MS (EI): 204 (17), 189 (36), 161 (27), 119 (61), 105 (64), 94 (64), 93 (88), 91 (85), 79 (82), 41 (100).

(+)-β-himachalene (αD20=239, neat, 1 dm):

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.73 (s, 3 H), 0.97 (s, 3 H), 1.35–1.58 (m, 4 H), 1.71 (s, 3 H), 1.74 (s, 3 H), 1.83–1.99 (m, 4 H), 2.40–2.49 (m, 1 H), 2.57–2.66 (m, 1 H), 2.89 (s, 1 H), 5.41 (s, 1 H).

MS (EI): 204 (30), 134 (32), 121 (33), 119 (100), 105 (47), 93 (39), 91 (60), 41 (55).

2,6,6,9-Tetramethylbicyclo[5.4.0]undec-8-ene (Fig. 1, **2**): (–)-α-Himachalene (1.039 g, 5.08 mmol) was dissolved in 60 ml ethanol and hydrogenated at room temperature in the presence of platinum(iv) oxide (497 mg). Hydrogen consumption was measured by using a mercury gas burette, and stalled at 1.3 eq. after 30 min stirring. The reaction was monitored by TLC. The reaction mixture was filtered through an analytical filter paper, the flask and the filter

were rinsed with ethanol, and the filtrate was evaporated *in vacuo* to obtain the product as colorless oil. Yield 1.049 g (100%), diastereomeric ratio 2*R*:2*S*≈9:1 (calculated from the integral values of the ^{1}H NMR signals of H-8).

Major 2*R* diastereomer: ^{1}H NMR ($CDCl_3$) δ=0.90 (s, 3 H, Me-6), 0.94 (d, ^3J=6.4 Hz, 3 H, Me-2), 0.96 (s, 3 H, Me-6), 1.18–2.14 (m, 13 H, H-1–7,10–11), 5.45 (m, 1 H, H-8).

Minor 2*S* diastereomer: ^{1}H NMR ($CDCl_3$) δ=0.90 (s, 3 H, Me-6), 0.92 (d, $^3J_{2,2a}$=7.1 Hz, 3 H, Me-2), 0.96 (s, 3 H, Me-6), 1.18–2.14 (m, 13 H, H-1–7,10–11), 5.52 ppm (m, 1 H, H-8).

Mixture of diastereomers: ^{1}H NMR (benzene-d_6) δ=0.95 (d, $^3J_{2,2a}$=6.6 Hz, 3 H, Me-2), 0.97 (s, 3 H, Me-6), 1.00 (s, 3 H, Me-6), 1.25 (m, 1 H), 1.30–2.00 (m, 14 H), 2.11 (m, 1 H), 5.56 (s, 1 H, H-8), 5.62 ppm (s, 0.17 H, H-8).

GC retention time of the major 2*R* diastereomer: 8.16 min; MS (EI): 206.1 (63, $M^{+\bullet}$), 163.0 (23), 150.0 (9), 134.9 (29), 120.9 (100); retention time of the minor 2*S* diastereomer: 8.25 min, *m/z*=206.0 (82%, $M^{+\bullet}$), 162.9 (24%), 150.0 (11%), 135.0 (30%), 120.6 (80%), 108.9 (61%), 92.9 (100%). R_f : 0.79 (*n*-pentane).

8,9-Dibromo-2,6,6,9-tetramethylbicyclo[5.4.0]undec-8-ene (Fig. 1, **3**): Bromination of **2** was carried out analogously to the procedure of Hofmann et al. (1994). Thus, the starting olefin (2.81 g, 13.6 mmol, 1 eq.) was dissolved in chloroform (50 ml), and the solution was cooled to 0–5°C. A solution of bromine (2.36 g, 14.8 mmol, 1.09 eq.) in chloroform (50 ml) was added dropwise over the period of 27 min, and the reaction mixture was stirred at 0–5°C for 2 h. The reaction mixture was evaporated *in vacuo* and the residue was dried. The crude product was used directly in the next step without further purification.

2,2,6,10-Tetramethylbicyclo[5.4.0]undeca-1(11),9-diene [(+)-(6R,7S)-himachala-9,11-diene] (Fig. 1, **4**): The reaction was carried out analogously to the procedure of Van Epp et al. (1981). Thus, the dibromide (1.094 g, 2.99 mmol, 1 eq.) was dissolved in dry tetrahydrofuran (THF, 1 ml) and a solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 929 mg, 6.10 mmol, 2.04 eq.) in dry THF (25 ml) was added. The reaction mixture was stirred for 2 hr at room temperature and was partitioned between dichloromethane (25 ml) and water (25 ml). The organic phase was separated and evaporated *in vacuo* and the product mixture was quickly dried. The product mixture was analyzed by GC-MS. The position of the double bonds was proven by a reaction with 4-phenyl-1,2,4-tetrazoline-3,5-dione.

The synthetic product contained the physiologically active (+)-(6*R*,7*S*)-himachala-9,11-diene (4%), *ar*-himachalene (5%), which is also emitted from feeding *P. striolata* males, and several other by-products that were not characterized. *ar*-Himachalene apparently is formed by aromatization of the six-membered ring during the didehydrobromination. Field tests in Taiwan (see section below) were performed with the raw product of the synthesis. For laboratory bioassays, (+)-(6*R*,7*S*)-himachala-9,11-diene was isolated by preparative gas chromatography with an Agilent 7890A GC instrument equipped with an HP5 capillary column (30 m×0.53 mm ID with 1.5 μm film thickness, Agilent) connected to a preparative fraction collector with a cryostatic trap cooler (PFC, Gerstel). The oven program was 40°C for 1 min, increased at 20°C/min to 120°C, then with 10°C/min to 210°C, and finally with 30°C/min to 300°C. The carrier gas was helium with a constant flow rate (4.8 ml/min). The obtained (+)-(6*R*,7*S*)-himachala-9,11-diene had a purity of 95% calculated from peak area of a GC-MS TIC. It was diluted in hexane to a concentration of 1 μg/μl for laboratory bioassays. (MS (EI+): 204.2 (30, $M^{+\bullet}$), 161.1 (24), 133.1 (100), 119.1 (78), 105.1 (72), 93.0 (28), 91.0 (39), 69.0 (18), 55.0 (21), 41.0 (29), GC-HRMS (EI) [M^+] ($C_{15}H_{24}$) calculated: 204.187801; found: 204.187019). (6*R*,7*S*)-Himachala-9,11-diene was characterized previously (incl. NMR and optical properties) by Muto et al. (2004).

Field Bioassays During Spring 2010, field bioassays were conducted at the experimental farm of AVRDC-The World Vegetable Center in Taiwan. Traps modified from Peng and Weiss (1992) served to assess the attractiveness of volatiles emitted by the host plant *B. juncea* and by *P. striolata* adults. *Brassica juncea* seedlings were grown to the second extended leaf in 11 cm diam plastic pots half filled with growing substrate (Blumenerdewerk Stender GmbH, Germany). Pots were covered with fine mesh, wrapped in a plastic bag and placed in a glass petri dish with a diameter of 15 cm and a height of 2 cm filled with soapy water. Beetles trapped in the soapy water were collected after 24 hr, counted, and sexed by inspection of the antenna and genitalia. Traps were arranged in a harvested crucifer field, which was previously infested with *P.striolata*, in a randomized complete block design with 15 replicates per experiment. The distance between traps in the block was 5 m, and blocks were 6 m apart.

Experiment 1 (February 23–25, 2010) The attractiveness of volatiles emitted from (a) intact *B. juncea* seedlings; (b) seedlings previously feeding-damaged by 20 *P. striolata* males; (c) seedlings previously feeding-damaged by 20 *P. striolata* females; (d) seedlings with 20 actively feeding females; and (e) seedlings with 20 actively feeding males, was assessed. Prior to the experiment, beetles were allowed to feed for 24 hr on the host plants in treatments (b) and (c). They were removed immediately before the experiment started.

Experiment 2 (February 25–27, 2010) The attractiveness of (a) moistened soil; (b) 20 males on moistened soil; (c) intact

seedlings with 20 males in a separate screened plastic vial with moistened tissue; was compared with (d) feeding-damaged seedlings (from 20 males for 24 hr) also separated from 20 males as in treatment (c); and (e) 20 actively feeding *P.striolata* males.

A field trapping experiment was conducted at the experimental farm of AVRDC-The World Vegetable Center from March 5 to 8 2010 to test the attractiveness of synthetic (+)-(6*R*,7*S*)-himachala-9,11-diene and AITC. Natural rubber septa (10×20 mm, Wheaton, Millville, NJ, USA) were used as pheromone dispensers. They were cleaned by sonification for 4 hr in dichloromethane and air-dried in a fume hood before use. The synthesis product containing (+)-(6*R*,7*S*)-himachala-9,11-diene was diluted in hexane to a concentration of about 5 μg/μl of the putative pheromone. A total of 250 μg (+)-(6*R*,7*S*)-himachala-9,11-diene was loaded onto each test septum. Control septa were treated with the same amount of hexane. The emission rate from pheromone septa was not tested. Soroka et al. (2005) determined the mean emission rate of (+)-(6*R*,7*S*)-himachala-9,11-diene from rubber septa loaded with 250 μg to be 11.8 μg/d in the laboratory at 27°C. Pure AITC (94% purity, Acros Organics) at a dose of 40 mg was applied in 0.5 ml PE tubes. Empty tubes served as control. Septa and opened PE tubes were placed close to each other in the center of sticky traps (20×25 cm sticky area, Jackson Traps, Jhen Yong Company, Taiwan). Traps were placed in a Chinese cabbage field with 1 m distance to infested host plants and arranged in a randomized complete block design with 10 replicates. The distance between traps in one block was 4 m and at least 6 m between blocks. Trap catches were evaluated once at the end of the experiment.

Experiment 3 Traps without bait were used as (a) control and compared to sticky traps baited with (b) 40 mg AITC; (c) 250 μg (+)-(6*R*,7*S*)-himachala-9,11-diene; and (d) 40 mg AITC combined with 250 μg of (+)-(6*R*,7*S*)-himachala-9,11-diene.

Two-Choice Bioassay Behavioral responses of *P. striolata* adults to natural and synthetic odors also were analyzed in a two-choice bioassay. Two traps were placed in a plastic container (295×230×185 mm). A wide hole was cut into the lid of the container and closed with fine mesh for air supply. Traps were transparent plastic tubes (height 10 cm, ID 4.5 cm) containing the test odor, and closed with a foam rubber plug. The plug was perforated in the center with a cut 1 ml pipette tip as entrance. The distance between traps was 10 cm, and positions were changed between replicates. Fifty starved *P. striolata* unsexed adults were placed inside the container and kept in an environmental chamber at 24°C with 65% humidity and L12:D12. The number of beetles that had entered each trap was counted after 24 hr.

Experiment 4 The bioassay set up was tested with the natural odor blend from (I) 25 male *P. striolata* feeding on a cut *B. rapa* var. *chinensis* leaf vs. an empty control, and (II) 25 male *P. striolata* vs. 25 female *P. striolata* both provided with a cut *B. rapa* var. *chinensis* leaf to feed on. Beetles and foliage representing the odor source were caged in transparent plastic tubes (height 5 cm, ID 3.3 cm) closed with a fine mesh within the traps.

Experiment 5 The attractiveness of (I) purified synthetic (+)-(6*R*,7*S*)-himachala-9,11-diene was tested by applying 5 μg of the compound on a piece of filter paper in test traps and the equivalent amount of pure hexane in control traps, and (II) 5 μg (+)-(6*R*,7*S*)-himachala-9,11-diene combined with 100 μg AITC was compared to the attractiveness of 100 μg AITC. Each treatment combination was replicated 10 times in Experiment 4, and 12 times in Experiment 5.

Statistics Statistical analyses were conducted with the software SAS Version 9.1 (SAS Institute 2001). Field data were log(x+1)-transformed and analyzed by analysis of variance (ANOVA) and *post-hoc* Tukey's HSD test. Data from the two-choice bioassays was analyzed by Wilcoxon signed rank test.

Results

Identification of Male-specific Compounds Volatile collections from male and female *P. striolata* feeding on cut *B. juncea* leaves were compared, and six male-specific sesquiterpenes A–F were identified. Compounds A, C, E, and F dominated the blend, (Table 1, Fig. 2a). The blend ratio was comparable between volatile collections and the mean total amount of detected sesquiterpenes was 18.4± 4.4 ng/male/d. Compound A was the most abundant in the blend (about 30%), and it was the only male-specific sesquiterpene eliciting an antennal response in females and males (Fig. 2b). It was identified as himachala-9,11-diene based on its mass spectrum (Bartelt et al., 2001). Muto et al. (2004) clarified the absolute configuration of several flea beetle derived sesquiterpenes by enantioselective synthesis and assigned the (+)-(6*R*,7*S*)-configuration to himachala-9,11-diene emitted by *P. cruciferae* and *Aphthona flava* (Guillebeau). This was shown to be the opposite configuration of the enantiomer (–)-(6*S*,7*R*)-himachala-9,11-diene present in *A. nordmanniana* (Bartelt et al., 2001). In our experiments, the himachala-9,11-dienes from *P. striolata* males and *A. nordmanniana* had the same retention time on an HP-5 column (Fig. 3c). However, himachala-9,11-dienes from both sources were separated by a retention time difference of 0.25 min by chiral stationary phase GC. Male

Table 1 *Phyllotreta striolata* male-specific compounds detected in volatile collections from 30 feeding males[a]

Male-specific compounds	Retention Index	Relative abundance(mean% of total±SD)	Method of identification	Assignment of stereochemistry
A (+)-(6*R*,7*S*)-Himachala-9,11-diene	1447	31.2±7.2	NIST[b], AR[c], Bartelt et al.[d]	chiral stationary phase GC, comparison to enantiomer from*Abies nordmanniana*
B (+)-α-Himachalene	1474	3.7±2.2	NIST, AR	comparison to (+)-α-himachalene from *S. undulata*
C *trans*-α-Himachalene	1494	24.8±3.0	Bartelt et al.	chiral stationary phase GC
D (+)-β-Himachalene	1524	5.0±1.9	NIST, AR	
E γ-Cadinene	1534	21.4±2.1	Bartelt et al.	
F (*R*)-*ar*-Himachalene	1559	15.9±5.3	Bartelt et al.	chiral stationary phase GC, *A. nordmanniana* (see above)

[a] Relative abundance of the single compounds in the blend is given in percent of the total male-specific sesquiterpene blend

Means were calculated from 10 volatile collections

[b] National Institute of Standards (NIST) mass spectral library

[c] Mass spectrum and retention time of authentic reference compound

[d] Mass spectrum published by Bartelt et al. (2001)

P. striolata, therefore, emit the (+)-(6*R*,7*S*)-himachala-9,11-diene enantiomer (Fig. 3a, b). The minor compound B was identified as α-himachalene based on its mass spectrum and retention time on the HP-5 column by comparison with the authentic reference compound (–)-α-himachalene isolated from *C. atlantica*. When analyzed on the chiral GC column, the retention time of the putative α-himachalene from *P. striolata* differed from the (–)-α-himachalene standard. The liverwort *Scapania undulata* (L.) is known to contain (+)-α-himachalene (Andersen et al., 1977), and a sample of *S. undulata* extract (donated by S. Garms; Dept. of Bioorganic Chemistry; MPI CE Jena) was compared with the male-specific sesquiterpenes. By comparison of retention times using a chiral GC column, compound B was identified as (+)-α-himachalene. The mass spectrum of compound C is similar to the mass spectrum of α-himachalene. However, the delayed retention time suggests that this compound is *trans*-α-himachalene, as previously described for *P. cruciferae*, and *Aphthona* spp. (Bartelt et al., 2001). Compound D was identified as β-himachalene by comparison with β-himachalene distilled from *C. atlantica* samples using standard and chiral stationary phase GC. Compounds E and F were identified as γ-cadinene and *ar*-himachalene, respectively, by comparison of their mass spectra with published mass spectra (Bartelt et al., 2001). γ-Cadinene and *ar*-himachalene also were found in headspace volatiles from *A. nordmanniana*. γ-Cadinene from the beetle and Nordmann fir did not separate on standard and chiral GC columns, suggesting that γ-cadinene from both sources have the same stereochemistry. *ar*-Himachalene from *P. striolata* and *A. nordmanniana* co-eluted on the non-polar column, but could be separated with chiral stationary phase GC by 1.4 min, and are therefore enantiomers. Enantioselective synthesis has clarified the absolute configuration of the leaf beetle-produced *ar*-himachalene to be (*R*) (Muto et al., 2004), while *ar*-himachalene isolated from different Abietoideae species has been determined to be (*S*) (Pandy and Dev, 1968; Muto et al., 2004). Previously, *ar*-himachalene

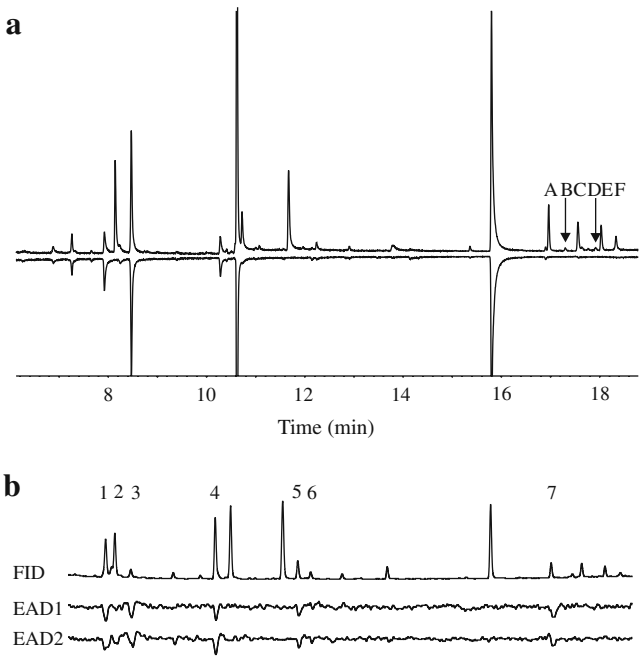


Fig. 2 Identification of male specific compounds and antennal responses to male and host plant volatiles: **a** Comparison of chromatograms (GC-MS) from male (*top*) and female (*inverted*) *Phyllotreta striolata* feeding for 12 hr on one cut *Brassica juncea* leaf. The sesquiterpenes A to F were not detectable in collections from females. **b** EAD responses of two male antennae. Seven compounds are physiologically active with compound 7 corresponding to the male-specific sesquiterpene A (Fig. 2**a**). The other compounds are the host plant volatiles (1) (*Z*)-3-hexen-1-ol, (2) 1-hexanol, (3) allyl isothiocyanate, (4) 3-butenyl isothiocyanate, (5) 4-pentenyl isothiocyanate, and (6) nonanal

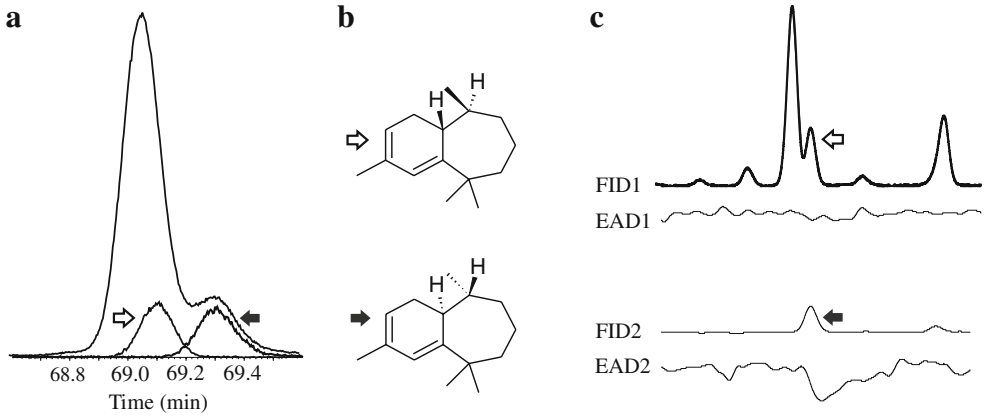


Fig. 3 Determination of the absolute configuration of himachala-9,11-diene emitted from male *Phyllotreta striolata*. **a** Overlay of gas chromatograms measured with a chiral column of (i) a co-injection of headspace samples from *Abies nordmanniana* and feeding male *P. striolata* (ii) headspace from *A. nordmanniana* (*white arrow*), (iii) headspace from feeding male *P. striolata* (*black arrow*). **b** white arrow: (–)-(6*S*,7*R*)-himachala-9,11-diene from *A. nordmanniana*, black arrow: (+)-(6*R*,7*S*)- himachala-9,11-diene from male *P. striolata*. **c** GC-EAD responses (HP-5 column) of female *P. striolata* antennae to headspace from *A. nordmanniana* (traces 1) and headspace from feeding male *P. striolata* (traces 2). The enantiomer present in the beetle extracts (*black arrow*) elicits an antennal response, while the one in *A. nordmanniana* headspace (*white arrow*) does not. The himachala-9,11-diene peak is preceded by ß-caryophyllene in the *A. nordmanniana* headspace extract

from both sources was considered to have the same configuration since both were reported to have a positive rotation (Pandy and Dev, 1968; Bartelt et al., 2001). However, Mori (2005) demonstrated that the optical rotation of (*R*)-ar-himachalene depends on the solvent in which it is measured. It is dextrorotatory in hexane, but levorotatory in chloroform, which led to the initial confusion.

Electrophysiology In GC-EAD experiments, the antennal responses of male and female *P. striolata* to host plant volatiles and male specific sesquiterpenes present in volatile collections from feeding *P. striolata* males were analyzed (Fig. 2b). Out of the six male-specific compounds, only (+)-(6*R*,7*S*)-himachala-9,11-diene (A), elicited EAD responses, and was detected by male and female antennae at levels<5 ng per GC-EAD run. The enantiomer (–)-(6*S*,7*R*)-himachale-9,11-diene from *A. nordmanniana* was not physiologically active (Fig. 3c). The male-specific compounds B to F were not EAD-active at levels present in headspace extracts. Furthermore, beetle antennae responded to several host plant volatiles. These included thee alkenyl glucosinolate hydrolysis products, allyl isothiocyanate, 3-butenyl isothiocyanate, and 4-pentenyl isothiocyanate, as well as the green leaf alcohols (*Z*)-3-hexenol and 1-hexanol. Since the synthetic (+)-(6*R*,7*S*)-himachala-9,11-diene could not be purified prior to field testing, the synthetic blend also was analyzed via GC-EAD. Synthetic (+)-(6*R*,7*S*)-himachala-9,11-diene was the only compound in the synthetic blend that elicited antennal responses in *P. striolata* males and females.

Field Bioassays Male *P. striolata* feeding on *B.juncea* seedlings attracted significantly more beetles (198) than any other treatment in Experiment 1 (Table 1). About the same number of conspecifics was attracted by volatiles emanating from intact seedlings (22); seedlings that had been damaged by either males (48) or females with beetles removed (38); or volatiles from seedlings with feeding females (31). Both sexes were almost equally attracted to the volatile blend from feeding males (54% females). In Experiment 2, males feeding on the host plants attracted significantly more beetles (220) than any other treatment. The number of beetles attracted by males on moistened soil (17); males separated from intact *B. juncea* plants (19); or males separated from feeding-damaged plants (23) was not significantly different from the number attracted by moistened soil alone (6) (Table 2). The sex ratio (58% females) of the beetles caught in traps baited with actively feeding male *P. striolata* was not different from Experiment 1.

Field Testing of (+)-(6R,7S)-himachala-9,11-diene Traps baited with (+)-(6*R*,7*S*)-himachala-9,11-diene alone did not attract more beetles (147) than control traps (129). AITC baited traps attracted significantly more beetles (259) than both control traps and traps baited with (+)-(6*R*,7*S*)-himachala-9,11-diene. However, traps with a combined stimulus of AITC and (+)-(6*S*,7*R*)-himachala-9,11-diene attracted significantly more beetles than any other treatment (416) (Table 3, Experiment 3).

Two-choice Laboratory Bioassay In Experiment 4, traps emitting the natural volatile blend from 25 male *P. striolata*

Table 2 Response of male and female *Phyllotreta striolata* to *P. striolata* adults and *Brassica* host plants

Treatment	No. *P. striolata* (mean±SD)[a]		Sex Ratio (M : F)
Experiment 1			
Intact *B. juncea*	1.5±1.0	b[b]	1 : 1.8
B. juncea feeding-damaged by 20 females	3.2±2.5	b	1 : 1.4
B. juncea feeding-damaged by 20 males	2.5±1.8	b	1 : 1.5
B. juncea with 20 feeding females	2.1±1.0	b	1 : 1.2
B. juncea with 20 feeding males	13.2±3.4	a	1 : 1.2
F=6.3 P<0.001			
Experiment 2			
Soil (Control)	0.4±0.6	b	1 : 5.0
Soil with males	1.1±1.3	b	1 : 1.4
Intact *B. juncea* plus 20 males	1.3±1.2	b	1 : 1.7
B. juncea feeding-damaged by 20 males plus 20 males	1.5±1.3	b	1 : 0.8
B. juncea with 20 feeding males	14.7±10.5	a	1 : 1.4
F=11.37 P<0.001			

[a] No. trapped/d in field bioassays at AVRDC-The World Vegetable Center, Taiwan

[b] Means followed by the same letters are not significantly different (P=0.05, Tukey HSD test)

feeding on one cut *B. rapa* var. *chinensis* leaf attracted (I) significantly more beetles (106) than control traps (15), as well as (II) significantly more beetles (86) than traps baited with 25 feeding females (20). In Experiment 5 (I), traps baited with 5 μg purified (+)-(6*R*,7*S*)-himachala-9,11-diene caught significantly more *P. striolata* (28) than control traps (3). (II) Similarly, *P. striolata* adults strongly preferred 100 μg AITC combined with 5 μg (+)-(6*R*,7*S*)-himachala-9,11-diene (36) in comparison to 100 μg AITC (2) (Table 4).

Table 3 Field trapping experiment of *Phyllotreta striolata* in response to synthetic (+)-(6*R*,7*S*)-himachala-9,11-diene alone and in combination with AITC

Treatment	Mean trap catch per day±SD	
Solvent control	4.3±1.7	c[a]
AITC	8.6±4.4	b
(+)-(6*R*,7*S*)-himachala-9,11-diene	4.9±2.6	c
AITC and (+)-(6*R*,7*S*)-himachala-9,11-diene	13.9±6.7	a
F=10.21 P<0.001		

[a] Means followed by the same letters are not significantly different (α=0.05, Tukey HSD test)

Discussion

Field bioassays revealed that volatiles emitted by *P. striolata* males feeding on host plants attract both sexes of the species. Active feeding or contact with the host plant is mandatory for the males to be attractive, since damaged host plants or male beetles alone, which had had the opportunity to feed on the host plant prior to the experiment, failed to attract conspecifics (Table 2). These findings match reports from diverse subfamilies of Chrysomelidae, where male-produced aggregation pheromones have been identified. Consistently, males release pheromonal compounds only while feeding on the host (Peng et al., 1999; Dickens et al., 2002; Cossé et al., 2002; Tóth et al., 2005; Zilkowski et al., 2006; Bartelt et al., 2006). However, from a physiological perspective, it is an open question whether actual feeding or contact with the host plant triggers the release of the aggregation pheromone.

By comparative chemical analyses, we identified six sesquiterpenes specifically emitted by feeding male *P. striolata* (Fig. 2a, Table 1). Among these compounds, only (+)-(6*R*,7*S*)-himachala-9,11-diene (A) elicited responses from *P. striolata* adult antennae, when using headspace samples in GC-EAD experiments. (+)-(6*R*,7*S*)-himachala-9,11-diene was synthesized from (–)-α-himachalene (Fig. 1) with known stereochemical configuration (Joseph and Dev, 1968). The configuration of the synthetic himachala-9,11-diene was identical to the corresponding compounds from male beetles based on retention times and mass spectra obtained with standard and chiral stationary phase GC-MS. Of the compounds present in the raw product of the synthesis, only (+)-(6*R*,7*S*)-himachala-9,11-diene, and none of the by-products, elicited antennal responses in *P. striolata* adults. In a field setting close to potential host plants the (+)-(6*R*,7*S*)-himachala-9,11-diene raw product enhanced the attractiveness of AITC, a known attractant for both sexes significantly, but was not behaviorally active on its own. In contrast, (+)-(6*R*,7*S*)-himachala-9,11-diene *per se* attracts *P. striolata* in a laboratory two-choice assay when offered as purified compound without other competing odor sources. However, when using natural odor sources, the number on non-responders dropped and more beetles were attracted to the natural volatile bouquet from *P. striolata* males feeding on *B. rapa* var. *chinensis* (Table 4, Experiment 4) compared to the numbers of beetles responding and being attracted to the synthetic pheromone compound alone or combined with AITC (Experiment 5). These data show that (+)-(6*R*,7*S*)-himachala-9,11-diene acts as aggregation pheromone of *P. striolata*. We cannot rule out that other compounds present in the raw product of the pheromone synthesis prevented the attractiveness of (+)-(6*R*,7*S*)-himachala-9,11-diene in the field. Nevertheless, this option is considered unlikely

Table 4 Two-choice laboratory bioassay of the response of *Phyllotreta striolata* to natural volatile sources and synthetic compounds

Treatment	Mean number of *P. striolata* per trap	Wilcoxon signed-rank test
Experiment 4		
B. rapa var. *chinensis* leaf with 25 males	10.6	Z=2,807
Control	1.5	P=0.005
B. rapa var. *chinensis* leaf with 25 males	8.6	Z=2.821
B. rapa var. *chinensis* leaf with 25 females	2	P=0.005
Experiment 5		
(+)-(6*R*,7*S*)-himachala-9,11-diene	2.33	Z=2.354
Solvent Control	0.25	P=0.018
(+)-(6*R*,7*S*)-himachala-9,11-diene and AITC	3	Z=2.995
AITC	0.17	P=0.003

since none of the other potentially relevant compounds present at the same or even higher concentration elicited physiological responses from the beetle antennae (data not shown). The combined data from field and laboratory experiments suggest that in a reduced environment the pheromone is attractive, while at a complex natural odor background the simultaneous presence of host plant volatiles, e.g., AITC, is required to trigger the behavioral response of *P. striolata* beetles to the aggregation pheromone. Moreover, the laboratory assays indicate that more plant and/or pheromone compounds than those identified to date are behaviorally active if offered as blend.

In the Alticinae, male-specific sesquiterpenoid compounds overlap among different species and genera. Of the six male-specific sesquiterpenes emitted by *P. striolata*, four (A, C, E, F; Table 1) were identified previously in male headspace extracts from the flea beetle species *P. cruciferae*, *Aphthona flava*, *A. czwalinae* (Weise), and *A. cyparissiae* (Koch) (Bartelt et al., 2001). Compounds A and F also were emitted by male *Epitrix fuscula* (Crotch) (Zilkowski et al., 2006). The behavioral function of these sesquiterpene blends was not further investigated in *Aphthona* spp. and *E. fuscula*. Bartelt and co-authors raised the hypothesis that the male-specific sesquiterpene blend constitutes the aggregation pheromone of the corresponding flea beetle species (Bartelt et al., 2001). However, in the European population of *P. cruciferae*, (+)-(6*R*,7*S*)-himachala-9,11-diene was as attractive as a synthetic blend of male-specific sesquiterpenes with AITC present in all treatments (Tóth et al., 2005). Moreover, traps baited with a sesquiterpene blend containing (+)-(6*R*,7*S*)-himachala-9,11-diene and AITC also attracted adults of congeneric species (Tóth et al., 2005). These results indicate that male aggregation pheromone compounds, individually or as blend, may attract flea beetles across species limits. Examples of cross-attraction of aggregation, but also sex pheromones, in congeneric species are known for several Coleoptera (Bartelt et al., 1995; Ayres et al., 2001; Wertheim et al., 2004). In the family Chrysomelidae, it has been shown that *Galerucella calmariensis* (L.) and *G. pusilla* (Duftschmidt) produce the same dimethylfuran lactone, and conspecifics of both species are attracted to the synthetic compound (Bartelt et al., 2006). In the scarab beetles *Melolontha melolontha* (L.) and *M. hippocastani* (Fabricius), three compounds, i.e., 1,4-benzoquinone, toluquinone, and phenol, are present in females of either species and function as sex pheromone. Male *Melolontha* beetles are attracted to females irrespective of the species. However, conspecific females are preferred when females from both species are offered simultaneously (Reinecke et al., 2006). Following these lines, aggregation pheromone blends may be attractive across species in flea beetles, but may nevertheless serve as species recognition cues in a natural choice situation. Adult *Phyllotreta* beetles may approach odor sources that resemble a species-specific olfactory search picture and finally decide for the best, i.e., species-specific stimulus. This hypothesis depends on the ability of the beetles to detect more than one compound of the male specific pheromone blend, and remains to be tested using more sensitive electrophysiological methodology.

Tóth et al. (2005) found physiological responses of *P. cruciferae* antennae to thee out of six male-specific compounds, (+)-(6*R*,7*S*)-himachala-9,11-diene being one of them. Zilkowski et al. (2006) demonstrated that himachalene-derived sesquiterpenes emitted by *E.fuscula* did not elicit physiological responses from the antennae, when used at the low concentrations present in headspace samples, but when stimulating with synthetic sesquiterpenes at a dose of 10 ng per compound, three compounds, again including (+)-(6*R*,7*S*)-himachala-9,11-diene, were active in EAD studies. In the male headspace samples used in the present study, minor compounds were present at a concentration below 1 ng/µl extract. Except for *ar*-himachalene, which was part of the raw product of the pheromone synthesis, physiological detection of other

male-specific sesquiterpenes from *P. striolata* may become apparent at higher doses only.

The glucosinolate hydrolysis products allyl-, 3-butenyl-, and 4-pentenyl isothiocyanate were present in the headspace of feeding-damaged *B. juncea* leaves, and they were all EAD-active. Any combination of these isothiocyanates with an aggregation pheromone, therefore, constitutes a reliable cue not only of a host plant, but a host plant already assessed and accepted by conspecifics. Correspondingly, AITC attracted *P. striolata* beetles *per se*, but adding the aggregation pheromone to the traps enhanced its attractiveness. AITC is also an attractant in other *Phyllotreta* species (Pivnick et al., 1992; Tóth et al., 2007). Even if combined with the aggregation pheromone, however, the evaporated amount of AITC required to attract flea beetles in our and in previous experiments (Soroka et al., 2005; Tóth et al., 2005) exceeds host plant emission rates by far (Pivnick and Jarvis, 1991). Moreover, glucosinolate contents and accordingly the amount of volatile hydrolysis products present in the headspace may vary to a wide extent in *Phyllotreta* host plants (Fahey et al., 2001; Verkerk et al., 2009). These findings lead to the hypothesis that a blend of host plant volatiles, glucosinolate hydrolysis products and/or other plant volatiles, constitutes the relevant stimulus that indicates a suitable host plant to *Phyllotreta* beetles. The high number of beetles attracted to the combined stimulus of beetles and host plants in the laboratory bioassay supports this hypothesis.

Interaction between insect pheromones and plant volatiles are well-documented in many species (Landolt and Phillips, 1997; Reddy and Guerrero, 2004; Seybold et al., 2006). For instance, female *Melolontha* cockchafers (Coleoptera: Scarabeidae) emit sex pheromones, which require the concomitant presence of green leaf volatiles to be behaviorally active (Ruther et al., 2000, 2002; Reinecke et al., 2002a, b). These examples demonstrate that rather unspecific plant volatiles may become an important part of the chemical orientation and communication if combined with more specific host or insect signals. Interestingly, the volatile green leaf alcohols 1-hexanol, and (*Z*)-3-hexenol were physiologically active in our electroantennographic experiments, and both compounds were more attractive than allyl isothiocyanate in olfactometer experiments with *P. cruciferae* (Gruber et al., 2009). However, when tested in the field individually or as binary combination, these leaf alcohols did not attract *P. striolata* adults, (data not shown). Combinations of (+)-(6*R*,7*S*)-himachala-9,11-diene with leaf volatiles were not tested during the past field season due to limited supply with the aggregation pheromone.

Another observation indicates that our current understanding of volatile glucosinolate hydrolysis products as kairomones for *P. striolata* may not fully reflect function and source of these compounds. When male *P. striolata* were allowed to feed on *Brassica* cultivars that do not produce alkenyl glucosinolates, relevant concentrations of AITC and/or 3-butenyl isothiocyanate were detected in the headspace, but were below detection limit when the plants were mechanically wounded (Beran, unpublished data). The beetles themselves thus are potential emitters of AITC synthesized from precursors acquired from the plant. The actual source of volatile isothiocyantes and the function of different host plant volatiles in the aggregation behavior of *P. striolata* are objects of ongoing research.

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Author contributions F.B. designed and performed experiments, analyzed natural extracts and synthetic products, and prepared the manuscript; I.M., C.U., R.S., and C.B. designed experiments, provided experimental facilities, insects, and acquired GTZ funding; C.V. and H.M. performed isolation of the precursor and reference substances and designed the synthetis pathway of A; J.S. and W.B. performed the synthesis of A and confirmed the structure of the product; A.R. and B.S.H. designed experiments and prepared the manuscript; MPG funding was provided though B.S.H.

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Chemical Profiles of Body Surfaces and Nests from Six Bornean Stingless Bee Species

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Abstract Stingless bees (Apidae: Meliponini) are the most diverse group of Apid bees and represent common pollinators in tropical ecosystems. Like honeybees they live in large eusocial colonies and rely on complex chemical recognition and communication systems. In contrast to honeybees, their ecology and especially their chemical ecology have received only little attention, particularly in the Old World. We previously have analyzed the chemical profiles of six paleotropical stingless bee species from Borneo and revealed the presence of species-specific cuticular terpenes— an environmentally derived compound class so far unique among social insects. Here, we compared the bees' surface profiles to the chemistry of their nest material. Terpenes, alkanes, and alkenes were the dominant compound groups on both body surfaces and nest material. However, bee profiles and nests strongly differed in their chemical composition. Body surfaces thus did not merely mirror nests, rendering a passive compound transfer from nests to bees unlikely. The difference between nests and bees was particularly pronounced when all resin-derived compounds (terpenes) were excluded and only genetically determined compounds were considered. When terpenes were included, bee profiles and nest material still differed, because whole groups of terpenes (e.g., sesquiterpenes) were found in nest material of some species, but missing in their chemical profile, indicating that bees are able to influence the terpene composition both in their nests and on their surfaces.

Key Words Stingless bees · Meliponines · Cuticular profile · Wax chemistry · Resin · Terpenes

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Introduction

Social insects— such as ants, termites, wasps and bees— live in large colonies with up to several thousands workers per colony. In order to communicate within colonies, they rely on a sophisticated communication system based on chemical cues (Blum et al. 1970). In addition to pheromones, waxy lipids on the cuticle (frequently referred to as cuticular profile or chemical profile) play an important role for communication (Blum et al. 1970; Buckner 1993), but also preserve insects from desiccation, cuticle abrasion, and infection (Lockey 1988; St. Leger 1995). The cuticular compounds comprise various chemical classes, with non-polar aliphatic compounds, such as methyl-branched alkanes, n-alkanes, and n-alkenes, dominating in ants (Hölldobler 1995; Endler et al. 2004), termites (Howard et al. 1982; Kaib et al. 2004), and social wasps (Espelie et al. 1994). In addition to these non-polar compounds, polar substances (with functional groups), such as alcohols, aldehydes, esters, and carboxylic acids, are found in various bee species (Ayasse et al. 1999; Paulmier et al. 1999; Fröhlich et al. 2000b; Abdalla et al. 2003; Jungnickel et al. 2004; Kerr et al. 2004; Mant et al. 2005; Nunes et al. 2008; Sramkova et al. 2008; Nunes et al. 2009). Moreover, we recently reported on yet another class of compounds in stingless bees from Borneo: besides non-polar aliphatic hydrocarbons, their cuticular profiles comprise large amounts of terpenes (Leonhardt et al. 2009), which most

likely are derived from plant resins collected (unpublished data).

In contrast to the environmentally derived terpenes in the chemical profiles of stingless bees, honeybees acquire compounds from self-produced comb wax (Breed et al. 1995, 1998, 2004). Therefore, the same compound classes can be found in comb wax and in the chemical profiles of honeybees, albeit in varying quantities (Blomquist et al. 1980; Fröhlich et al. 2000b; Breed et al. 2004). However, direct comparisons between bee profiles and nest material (such as comb wax) rarely have been made (but see Blomquist et al. 1980; Fröhlich et al. 2000b), which is surprising given that interactions between the bees and their nest material are supposed to play an important role in the origin of recognition cues (Breed et al. 1995, 1998, 2004). Bees even are able to distinguish between wax of their own and a foreign colony (Fröhlich et al. 2000a; Hepburn et al. 2010).

Here, we compared nest material and chemical profiles of six stingless bee species from Borneo. The chemical profiles of these bees strongly differ among species, with regard to both resin-derived terpenoid compounds and genetically determined non-terpenoid compounds (aliphatic compounds) (Leonhardt et al. 2009). Resin is the main nest building material in stingless bees, but it frequently is mixed with self-produced wax (Roubik 1989, 2006). Because stingless bees have both resin-derived compounds and genetically determined aliphatic hydrocarbons on their cuticle (Leonhardt et al. 2009), we asked whether they simply acquire their cuticular compounds from their nesting substrate, which would result in similar bee and nest profiles. The bees alternatively might be able to exclude or modify nest compounds before acquiring them on their body surface or produce cuticular profiles independent of their nest profiles. In this case, the chemical composition of the bees' body surfaces and their nest material might differ in resin-derived and/or genetically determined compounds.

Methods and Materials

Study Sites and Bees Bee specimens and nest material were collected at the Danum Valley Conservation Area (DVC: Sabah, 4°55′N 117°40′E, 100 m asl) and the Kabili Sepilok Reserve (KSR: Sabah, 5°54′N, 118°04′E, 20–120 m asl) in Sabah, Borneo (Malaysia). Sampling was performed in February and March 2007. KSR covers an area of 4294 ha of coastal dipterocarp and mangrove forest (Fox 1973) surrounded by oil palm plantations. DVC represents one of the major remaining patches of Sabah's primary lowland dipterocarp rainforest (43 800 ha) (Marsh and Greer 1992). The two sites have a typical equatorial rainforest climate with a mean annual temperature of 26–30°C and yearly rainfall of 2600–3000 mm (Fox 1973).

Eltz (2004) and Dworschak and Blüthgen (2010) found 15 stingless bee species (species and genus names as in Moure 1961) in DVC, whereas 15 to 20 species can be found in KSR according to specimen collections held by the Forestry Research Centre in Sepilok and our own studies (Leonhardt and Blüthgen 2009).

Sampling of Bees and Nest Material We caught departing workers from 29 colonies belonging to six species. These were *Tetragonula fuscobalteata, T. geissleri, T. melanocephala, Tetragonilla collina, Pariotrigona pendleburyi,* and *Lepidotrigona terminata*. Sixteen colonies were located in DVC and 13 in KSR (see also Leonhardt et al. 2009; note that *Tetragonula melina* was excluded from this study because nest material could not be obtained from this species). Bees were caught at their colonies' nest entrances by putting a clean transparent plastic bag over the nest entrance tube.

Nest material was obtained from 15 of the 16 colonies from DVC (comprising all 6 species), by breaking of small pieces of the nest entrance tube (max. ~1 mg). To test whether old and new nest material had the same chemical composition, we returned to 6 nests (including colonies from all species except *Tetragonula fuscobalteata*) after 1–9 d and additionally collected fresh, recently added nest material. Only fresh nest material was collected from two *Tetragonula geissleri* colonies.

Extraction and Chemical Analysis The bees were killed in a freezer. Dead bees and nest material collected were transferred into 2 ml sample vials containing 1.5 ml hexane for extraction. Bees were extracted for 10 min and then discarded; nest material was kept in hexane for the rest of the analysis. Extracts from bees and nest materials were analyzed by a Hewlett Packard HP 6890 Series GC System coupled to a Hewlett Packard HP 5973 Mass Selective Detector (Agilent Technologies, Böblingen, Germany) as described in Leonhardt et al. (2009).

Compounds found in the extracts were characterized by their mass spectra and retention times. Compounds with identical mass spectra and retention times were regarded as the same substance. Compound classes characterized comprised alkanes, aldehydes, alcohols, esters as well as mono-, sesqui-, and triterpenes. They were (tentatively) identified by comparison with three commercially available mass spectra libraries (Wiley 275, NIST 98 and Adams EO library 2205) and with compounds from dipterocarp tree resins known to comprise mono-, sesqui-, and triterpenes (see also Leonhardt et al. 2009). Compounds were confirmed by synthetic standards if standards were available (Sigma-Aldrich, Munich, Germany).

Statistical Analysis Prior to analyses, we condensed the dataset by removing trace compounds (for which mass

Table 1 Proportion of substance classes in hexane extracts of bee profiles (profile) and nest material (nest) from six stingless bee species*

	Tetragonilla collina		*Tetragonula fuscobalteata*		*T. geissleri*		*T. melanocephala*		*Pariotrigona pendleburyi*		*Lepidotrigona terminata*		
	profile	nest	profile	nest	profile	nest	profile	nest	profile	nest	profile	nest	*N2*
monoterpenes	0.03%	–	0.03%	–	6.49%	14.84%	0.06%	–	0.06%	–	0.02%	0.03%	8
sesquiterpenes	34.61%	65.41%	3.36%	0.06%	6.14%	14.03%	1.90%	50.30%	9.17%	24.20%	0.87%	3.81%	104
triterpenes	5.33%	18.45%	66.36%	78.05%	32.14%	14.35%	70.56%	33.14%	7.01%	48.87%	17.52%	64.21%	59
alkanes	10.74%	1.12%	8.41%	11.28%	2.92%	0.31%	14.31%	0.69%	15.38%	2.66%	23.63%	16.96%	13
methyl alkanes	0.04%	–	0.67%	–	–	–	0.18%	–	–	–	0.79%	–	3
alkenes	0.17%	–	9.56%	0.52%	7.95%	0.93%	2.06%	0.05%	0.10%	–	40.45%	2.17%	19
alkadienes	–	–	0.01%	–	2.98%	0.21%	–	–	–	–	5.47%	0.28%	6
ester	26.37%	0.78%	0.30%	–	0.73%	–	0.05%	–	2.54%	–	–	–	11
carboxylic acids	0.23%	–	–	–	–	–	–	–	–	–	–	–	1
alcohols	0.13%	–	0.31%	–	–	–	0.08%	0.08%	–	–	–	–	3
aldehydes	–	–	0.12%	–	0.01%	–	0.72%	–	–	–	–	–	2
lactones	–	–	–	0.26%	–	–	–	–	–	–	–	–	3
unidentified substances	16.87%	10.81%	5.63%	8.01%	36.51%	51.12%	5.82%	9.70%	62.31%	16.91%	7.19%	9.29%	76
N1	103	118	72	62	88	109	71	139	32	80	47	80	

N1=number of compounds per species

N2=total number of compounds found for a given substance class across species

*Proportions are obtained by dividing the peak area of a substance class by the total peak area of all compounds in a species (colonies pooled).

spectra could not be characterized) as well as compounds that accounted for less than 5% of the total peak area across all samples. The analysis is based on a total of 309 compounds, with 253 found in nest material and 195 in bee profiles. The compounds were quantified as proportions by dividing the peak area of each single compound by the total area of all peaks remaining after having condensed the dataset.

First, we analyzed species specific differences in the chemical composition of nests. Then, we compared nest materials and bee profiles to investigate whether bees chemically resemble their nests. Analyses were performed for all compounds, only terpenoid compounds, and only non-terpenoid compounds to distinguish between the contributions of resin-derived terpenoids and genetically determined compounds. Two-dimensional NMDS (non-metric dimensional scaling) based on Bray-Curtis distances of the proportions of each compound (start configuration: PCoA, 1000 iterations) was used to produce ordination figures. Groups were compared by an "Adonis" test (R Statistical software 2.9.2, vegan package; command for a randomization-based analysis of dissimilarities), based on the Bray-Curtis distance matrix of the proportions of each compound.

If surface profiles of bees merely mirrored their nest profiles, chemical differences between bees and their nest of origin should be less pronounced than between bees and nests of different colonies. To test whether chemical differences were more pronounced within nests and/or bee profiles or between nests and the equivalent bee profiles, we compared the mean Bray-Curtis distances for the two bee species with the largest sample sizes (*T. fuscobalteata* and *T. melanocephala*).

Results

Nest material of the six stingless bee species analyzed contained large amounts of resin-derived terpenes (particularly sesqui- and triterpenes), as well as wax-derived aliphatic compounds (Table 1). The chemical composition of nests from different species strongly differed both qualitatively and particularly quantitatively (Adonis: R^2=0.90, P<0.001; Fig. 1a; Table 1). Differences among species were similarly pronounced when only terpenoids were included in the analyses (Adonis: R^2=0.90, P<0.001; Fig. 1c) and slightly less for non-terpenoids (Adonis: R^2=0.86, P< 0.001; Fig. 1b). We found no differences in the chemical composition between old and fresh nest material (Adonis: R^2=0.02, P=0.98; Fig. 1).

One hundred and forty compounds were shared by both nest material and bee profiles, whereas 114 compounds

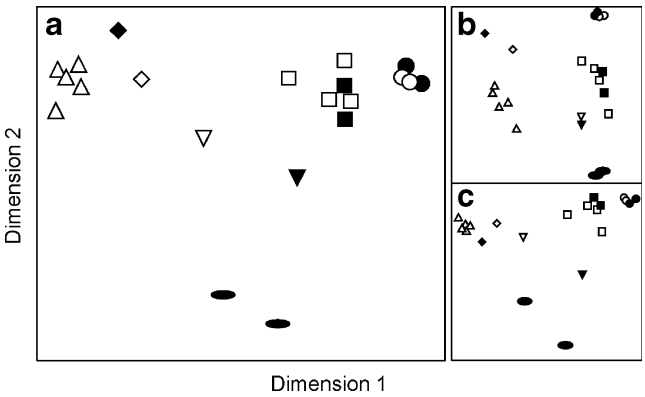


Fig. 1 Similarity in the chemical composition of nest material from six stingless bee species including (**a**) all compounds, (**b**) only non-terpenoid compounds, and (**c**) only terpenoid compounds (each symbol represents one colony). NMDS ordination based on Bray-Curtis distances: triangles *Tetragonula fuscobalteata*, diamonds *Lepidotrigona terminata*, triangles upside down *Pariotrigona pendleburyi*, squares *T. melanocephala*, circles *Tetragonilla collina*, ellipses *T. geissleri*; open symbols indicate old nest material and filled symbols indicate fresh nest material

were exclusively found in nest material and 55 exclusively in bee profiles. When nest material and bee profiles were combined in one analysis, both nests and bee profiles still could be discriminated (Adonis: all compounds: R^2=0.90, P<0.001; Fig. 2a; non-terpenoids: R^2=0.88, P<0.001; Fig. 2b; terpenoids: R^2=0.86, P<0.001; Fig. 2c). Nest and bee profiles were more similar chemically to one another than nests and their equivalent bee profiles, in both 1) *T. fuscobalteata* (mean Bray-Curtis distances ± SD between bee profiles: 0.20±0.13; between nest profiles: 0.19±0.06; between nest and equivalent bee profiles: 0.32±0.07) and 2) *T. melanocephala* (between bee profiles: 0.22 ± 0.03; between nest profiles: 0.33±0.07; between nest and equivalent bee profiles: 0.74±0.08). The same trend was observed for the Bray-Curtis distance matrices of only terpenoid compounds (*T. fuscobalteat*a: between bee profiles: 0.18±0.14; between nest profiles: 0.15±0.07; between nest and equivalent bee profiles: 0.22±0.10; and *T. melanocephala*: between bee profiles: 0.16±0.05; between nest profiles: 0.31± 0.07; between nest and equivalent bee profiles: 0.75±0.10) and for non-terpenoid compounds (*T. fuscobalteat*a: between bee profiles: 0.27±0.12; between nest profiles: 0.32±0.09; between nest and equivalent bee profiles: 0.62±0.04; *T. melanocephala*: between bee profiles: 0.32±0.10; between nest profiles: 0.38±0.15; between nest and equivalent bee profiles: 0.72±0.10).

For terpenoids, bee and nest profiles with sesquiterpenes and without sesquiterpenes were clearly separated (Fig. 2c), but discrimination was less pronounced for bee and nest profiles that solely contained triterpenes (Adonis: R^2=0.67, P<0.001; Fig. 2c). Notably, sesquiterpenes were present in hexane extracts of both nest material and bee surfaces of

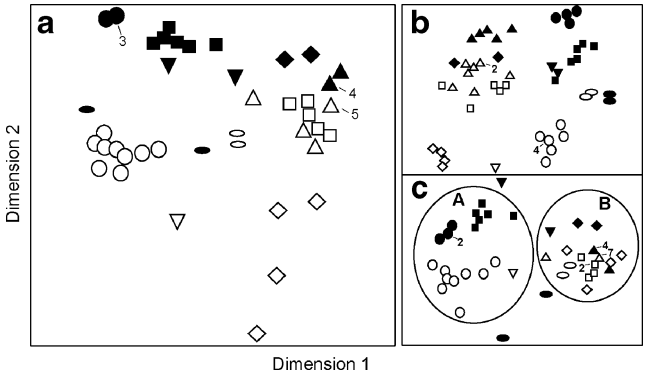


Fig. 2 NMDS of chemical compounds from hexane extracts of nest material and bee profiles combined of six bee species including (**a**) all compounds, (**b**) only non-terpenoid compounds, and (**c**) only terpenoid compounds (each symbol represents one colony); symbol codes as in Fig. 1; filled symbols indicate nest material and open symbols indicate bee profiles. Numbers indicate where several symbols were entirely overlapping. The two circles (in 2c) comprise samples with sesquiterpenes (*A*) and without sesquiterpenes (*B*)

some species (e.g., *T. collina*) (Fig. 3a, c, Table 1), whereas they were found only in trace amounts on the body surfaces of other species (e.g., *T. melanocephala*), while present in their nest material (Fig. 3b, d, Table 1).

Discussion

The chemical composition of nest material from six Bornean stingless bee species strongly differed both qualitatively and quantitatively. These differences prevailed in freshly collected nest material, indicating that the chemical composition remained constant over time. Moreover, bee profiles did not merely mirror the chemical profiles of their nests, because bee and nest profiles were clearly separated by their chemical composition. Furthermore, chemical distances were more pronounced between bees and their nests of origin than within bees and/or within nests from different colonies. Discrimination was about equally accurate when analyses were confined to either terpenoid or non-terpenoid compounds. The least discrimination was revealed for surface profiles and nests with triterpenes as sole terpene group, indicating that triterpenes were qualitatively and quantitatively similar across species' nests and surface profiles.

Besides terpenes, bee surfaces comprised alkanes, alkenes, branched alkanes, and alkadienes as well as compound classes with functional groups (such as esters or alcohols) (see also Leonhardt et al. 2009), whereas nest material mainly contained terpenes, alkanes, and alkenes. These findings are in accordance with studies of Milborrow et al. (1987) and Blomquist et al. (1985) that also revealed non-polar hydrocarbons and compounds from exogenous materials (terpenes) as dominant compound groups in the nesting substrate of stingless bees. Hence, genetically determined non-terpenoid compounds of stingless bees comprise mainly, partly even exclusively, non-polar aliphatic hydrocarbons (n-alkanes, alkenes and branched alkanes) and only few compounds with functional groups (see also: Abdalla et al. 2003; Jungnickel et al. 2004; Kerr et al. 2004; Nunes et al. 2008, 2009; Leonhardt et al. 2009). By contrast, honeybees have non-polar compounds and compounds with functional groups in equal quantities both on their body surfaces and in their nest material (comb wax) (Blomquist et al. 1980; Francis et al. 1985, 1989; Fröhlich et al. 2000b). Moreover, honeybees more closely resemble their nest material than do stingless bees (Blomquist et al. 1980; Fröhlich et al. 2000b). Besides genetically

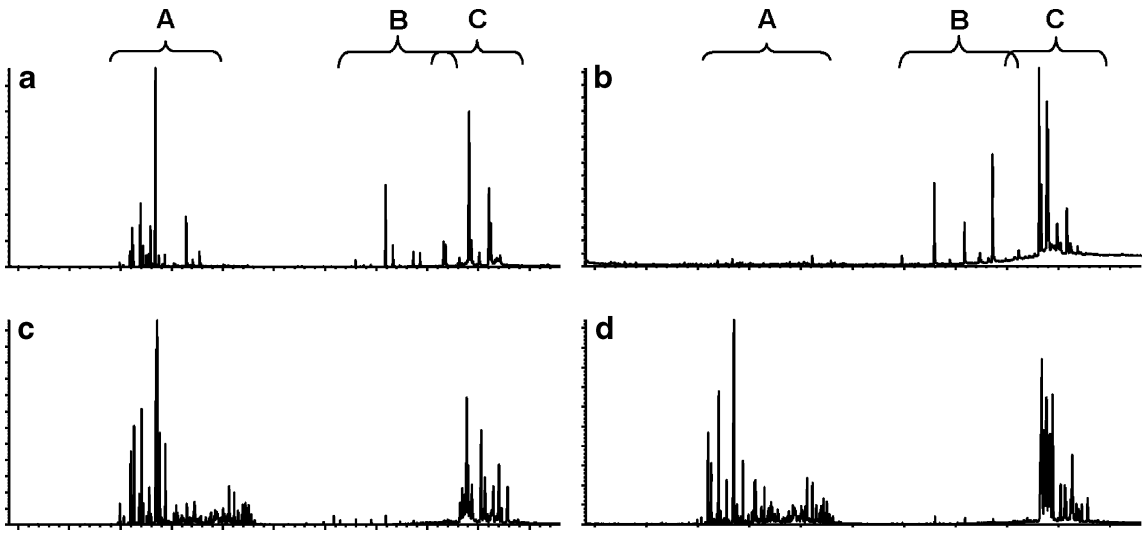


Fig. 3 Chromatograms of hexane extracts from (**a**) the chemical profile of a *Tetragonilla collina* worker, (**b**) the chemical profile of a *Tetragonula melanocephala* worker, (**c**) nest material of *T. collina*, and (**d**) nest material of *T. melanocephala*. Capital letters above chromatograms indicate substance classes of peaks/compounds: *A* mainly sesquiterpenes, *B* non-terpenoid compounds, and *C* triterpenes and non-terpenoid compounds

determined aliphatic hydrocarbons, the stingless bee species studied here have large amounts of resin-derived terpenes (especially sesqui- and triterpenes) both on their body surfaces and in their nests. This compound group is completely missing in surface profiles of honeybees (let alone any other social insect).

Because most substances (e.g., terpenes) were found in both nest material and surface profiles of stingless bees, compound transfer from nests to surfaces appears likely and would be in accordance with findings in honeybees, whose cuticular surfaces are influenced by compounds derived from beeswax (especially fatty acids and esters) (Breed et al. 1995, 1998, 2004). However, in stingless bees, resin-derived terpenes are not passively transferred from their nest material to their surface, because, in some species (e.g., *T. melanocephala*), one group of terpenes (sesquiterpenes) is found in nests, but completely lacking on body surfaces. Such a lack of sesquiterpenes suggests that stingless bees are able to actively influence the chemical composition of compounds in nests and on their surface by, for instance, excluding a whole group of terpenes. This idea is supported further by the highly species-specific distribution of different groups of terpenes among surface profiles of different bee species (Leonhardt et al. 2009). The species-specific terpene profiles contrast with the bees' generalistic resin collection behavior where all bee species largely visit the same set of tree species (unpublished data). Moreover, even nest material of different species can be discriminated based on terpenes alone (with chemical compositions remaining constant over time), rendering a purely opportunistic inclusion of resin-derived compounds in nest material unlikely. It appears most likely that resin and thus resin-derived compounds are processed prior to nest building, because resin foragers do not alter the resin collected directly at resin wounds (unpublished data). How stingless bees process plant resins remains as yet uninvestigated. They may be able to modify them by using specific enzymes or with the help of mutualistic microbes.

Stingless bees respond to terpenes, as they use these compounds to locate and re-recognize resin sources (wounds) at trees (Leonhardt et al. 2010b). Moreover, adding sesquiterpenes to the surface profile of species that lack sesquiterpenes themselves reduces aggression in these species, indicating that terpenes affect the bees' recognition system (Leonhardt et al. 2010a). Whether terpenes are involved further in nestmate recognition remains to be tested, but the species-specific distribution in both the bees' surface profiles and their nests suggests that terpenes represent more than pure contamination by plant resins.

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Novel Components of the Sex Pheromones Produced by Emerald Moths: Identification, Synthesis, and Field Evaluation

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Abstract The subfamily Geometrinae (Lepidoptera: Geometridae) includes many species called emerald moths. Based on our recent finding of novel polyenyl compounds, including a double bond at the 12-position from two geometrine species, *Hemithea tritonaria* and *Thalassodes immissaria intaminata*, (6*Z*,9*Z*,12*Z*)-6,9,12-trienes and (3*Z*,6*Z*,9*Z*,12*Z*)-3,6,9,12-tetraenes with a C_{17}–C_{20} straight chain were synthesized and analyzed by GC-MS. The 6,9,12-trienes, which were prepared by a double Wittig reaction between two alkanals and an ylide derived from (*Z*)-1,6-diiodo-3-hexene, characteristically produced fragment ions at *m/z* 79, 150, and M–98. The 3,6,9,12-tetraenes, which were prepared by a coupling between (*Z*)-3-alkenal and an ylide derived from (3*Z*,6*Z*)-1-iodo-3,6-nonadiene, showed fragment ions at *m/z* 79, 148, and M–96. These diagnostic ions were useful to distinguish these compounds from other known polyenyl pheromones, such as 4,6,9- and 6,9,11-trienes and 1,3,6,9-tetraenes. With reference to the GC-MS data, pheromone extracts of other species in Geometrinae inhabiting the Iriomote Islands were analyzed, and the 6,9,12-trienes were identified in the pheromone gland extracts of *Pamphlebia rubrolimbraria rubrolimbraria* and *Maxates versicauda microptera*. Furthermore, a field evaluation of the synthetic polyenes in a mixed forest of Tokyo revealed the following new male attractants for emerald moths: *Idiochlora ussuriaria* by a C_{17} 6,9,12-triene and *Jodis lactearia* by a C_{20} 3,6,9,12-tetraene, indicating the characteristic chemical structures of Geometrinae pheromones. On the other hand, through reexamination of the pheromone extract of *H. tritonaria*, (3*E*,6*E*)-α-farnesene was identified as an electrophysiologically active component in addition to the C_{17} 6,9,12-triene. The binary mixture attracted more males than the single component lure baited with the triene in the Iriomote Islands.

Key Words Female sex pheromone · Lepidoptera · Geometridae · Geometrinae · 6,9,12-Triene · 3,6,9,12-Tetraene · α-Farnesene · Male attraction

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Introduction

Lepidopteran sex pheromones, which have been identified from more than 600 species (Ando, 2010; El-Sayed, 2010), are divided primarily into two groups according to their chemical structure (Ando et al., 2004). Unsaturated C_{10}–C_{18} fatty alcohols and their derivatives compose Type I pheromones, and Type II pheromones consist of C_{17}–C_{23} polyunsaturated hydrocarbons and their epoxy derivatives. After the first discovery of a Type II pheromone from an Arctiidae species, *Estigmene acrea* Drury (Hill and Roelofs, 1981), (6*Z*,9*Z*)-6,9-dienes, (3*Z*,6*Z*,9*Z*)-3,6,9-trienes, and the epoxy derivatives were identified from many species in the relatively derived families, i.e., Geometridae, Noctuidae, Arctiidae, and Lymantriidae (Ando, 2010; El-Sayed, 2010). The dienes and trienes are believed to be biosynthesized starting from (9*Z*,12*Z*)-9,12-octadecadienoic acid (linoleic acid) and (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienoic acid (linolenic acid), respectively, by chain elongation and decarboxylation for the polyenes with an odd numbered chain

(Jurenka, 2004; Ando et al., 2008) and additional α-oxidation for the polyenes with an even numbered chain (Goller et al., 2007). In addition to these compounds, polyenes modified by additional desaturation at the 1-, 4-, or 11-position and the epoxy derivatives have been found (Ando et al., 2004; Ando, 2010; El-Sayed, 2010). Based on these identifications, we systematically synthesized candidates of the Type II pheromones and, by random screening tests, discovered new instances of male attraction in several geometrid and noctuid species (Ando et al., 1993, 1995; Yamamoto et al., 2008).

Geometridae is subclassified into several subfamilies, including Larentiinae, Ennominae, and Geometrinae. Sex pheromones have been studied only in species of the former two subfamilies (Ando, 2010; El-Sayed, 2010) and not in those in Geometrinae, which includes many species, such as emerald moths, with green wings. Thus, it is highly possible to find new pheromone compounds from the species in the Geometrinae. We collected male and female emerald moths using a light trap in the subtropical Iriomote Islands, and successfully identified novel pheromone components, (6*Z*,9*Z*,12*Z*)-6,9,12-octadecatriene from *Hemithea tritonaria* Walker and (3*Z*,6*Z*,9*Z*,12*Z*)-3,6,9,12-icosatetraene from *Thalassodes immissaria intaminata* Inoue (Yamakawa et al., 2009). This identification revealed that desaturation at the ω12-position of biosynthetic acyl precursors occured, at least in the above two species, establishing a species-specific pheromone system. There are about 2,300 Geometrinae species in the world, 86 species of which are distributed in Japan. It would be interesting to determine whether such desaturation is characteristic of the pheromones of Geometrinae species. To answer this question, we started synthesis of (6*Z*,9*Z*,12*Z*)-6,9,12-trienes and (3*Z*,6*Z*,9*Z*,12*Z*)-3,6,9,12-tetraenes with a C_{17}–C_{20} straight chain and accumulated their GC-MS data. Using the obtained spectral information, we analyzed the sex pheromones of other Geometrinae females, and the (6*Z*,9*Z*,12*Z*)-6,9,12-trienes were detected in pheromone gland extracts of two additional species, *Pamphlebia rubrolimbraria rubrolimbraria* Guenée and *Maxates versicauda microptera* Inoue. Next, we utilized the synthetic compounds in field surveys, and found new male attractants for two other species, *Idiochlora ussuriaria* Bremer and *Jodis lactearia* Linnaeus. On the other hand, it has been reported that *H. tritonaria* females produce an unknown pheromone component (Yamakawa et al., 2009). We reexamined the GC-MS data, and the compound is now assigned to be (3*E*,6*E*)-α-farnesene, which has not been detected in pheromone gland extracts of other species. The role of the sesquiterpene in the mating communication of *H. tritonaria* was evaluated in the field. This paper deals with unique communication systems developed by these five emerald moth species.

Methods and Materials

Insects and Pheromone Extraction Adults of two Geometrinae species, *Pamphlebia rubrolimbraria rubrolimbraria* and *Maxates versicauda microptera*, were collected with a black light in a mixed forest area including many evergreen broad-leaved trees in the Iriomote Islands, which are located in a subtropical zone (24.29°N, 123.86°E). The collection was carried out from 17:00 to 23:00 on several days in 2008 and 2009. The captured moths were placed separately in small glass vials and sent to the laboratory of TUAT in Tokyo. Eggs were obtained from the mated females of *P. r. rubrolimbraria*, and the larvae were reared on a semi-synthetic diet (Insecta LF, Nippon-Nosan-Kogyo Co., Yokohama, Japan) under a 16:8 h L:D cycle at 25°C. The abdominal tips of the virgin females were excised 1 h after the beginning of scotophase, and soaked in hexane (100 μl/tip) for 15 min to extract the pheromone. In the case of *M. v. microptera*, a pheromone extract was prepared from wild females.

Instruments for Analysis and Purification Gas chromatography with an electroantennographic detector (GC-EAD) was used to identify physiologically active compounds in female gland extracts. An HP-5890 Series II GC (Hewlatt-Packard, Wilmington, DE, USA) was equipped with a DB-23 column (0.25 mm ID, 30 m length, 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA). The column temperature was programmed from 50°C for 2 min, 10°C/min to 160°C, and 4°C/min to 220°C. The effluent from the column was split 1:1 into two lines, which led to a flame ionization detector (FID) and EAD (Inomata et al., 2005). GC-mass spectrometry (GC-MS) in the electron impact (EI) ionization mode (70 eV) was achieved using an HP-5975 MS system (quadrupole type, Hewlett-Packard) equipped with the same DB-23 column operated at the same temperatures as the GC-EAD. The HPLC system employed a pump (PU-980, JASCO), an RI detector (RI-98SCOPE, Labo System, Tokyo, Japan), an integrator (807-IT, JASCO), and an ODS column (Senshu Pack ODS-SH, 0.8 cm ID×15 cm; Senshukagaku, Tokyo, Japan). As the eluent, 5% H_2O in methanol was used at a flow rate of 1.0 ml/min for purification of synthetic polyenes.

Chemicals The chemical structures of polyunsaturated hydrocarbons are abbreviated as follows: Z = (*Z*)-double bond, number before the hyphen = position of the double bond, number after the hyphen = carbon number of the straight chain, and H = compound without terminal functional group. For example, (5*Z*,8*Z*,11*Z*)-5,8,11-heptadecatriene is abbreviated as Z6,Z9,Z12-17:H. The synthesis of Z6,Z9,Z12-18:H and Z3,Z6,Z9,Z12-20:H has been reported briefly (Yamakawa et al., 2009). The other trienes and tetraenes with a C_{17}–C_{20}

chain were prepared in a similar manner, as shown in Fig. 1. (3*E*,6*E*)-α-Farnesene (>98% purity) used for a field test was prepared by HPLC purification of a sample purchased from the Sigma-Aldrich Company. This sample included geometrical isomers of α-farnesene (3,7,11-trimethyl-1,3,6,10-dodecatetraene) and β-farnesene (7,11-dimethyl-3-methylene-1,6,10-dodecatriene), which also were isolated and used as an authentic standard of a GC-MS analysis after structure analysis by NMR. Details of the isolation and characterization will be reported elsewhere.

(6Z,9Z,12Z)-6,9,12-Trienes (Scheme A) Z6,Z9,Z12-17:H was synthesized starting from 3-hexyne-1,6-diol (**1**), which was converted into 1,6-diiodo-(*Z*)-3-hexene (**2**) by partial hydrogenation and iodination (Yamamoto et al., 2008). With the phosphonium salt of **2**, bis-ylide (**3**) was prepared (Pohnert and Boland, 2000) and treated with a 1:1 mixture of pentanal and hexanal to yield the objective C_{17} triene mixed with C_{16} and C_{18} trienyl by-product. Purification of Z6,Z9,Z12-17:H was achieved by preparative HPLC with an ODS column. In the same manner, Z6,Z9,Z12-19:H was synthesized by double Wittig coupling of **3** with a 1:1 mixture of hexanal and heptanal, and Z6,Z9,Z12-20:H was synthesized with a 1:1 mixture of hexanal and octanal. Undesired byproducts were removed by the preparative HPLC. ^{1}H NMR δ ppm: 0.98 (6H, t, *J*=7 Hz), ~1.3 (10H–16H for C_{17}–C_{20} trienes, m), 2.06 (4H, dt, *J*=7, 7 Hz), 2.81 (4H, dd, *J*=6, 6 Hz), 5.37 (6H, m). ^{13}C NMR δ ppm: Z6,Z9,Z12-17:H, 14.0 (C-17), 14.1 (C-1), 22.4 (C-16), 22.6 (C-2), 25.6 (× 2, C-8, 11), 27.0 (C-14), 27.2 (C-5), 29.4 (C-4), 31.5 (C-3), 31.9 (C-15), 127.67, 127.69 (C-7, 12), 128.3 (× 2, C-9, 10), 130.36 (C-13), 130.42 (C-6); Z6,Z9,Z12-18:H, 14.1 (C-1, 18), 22.6 (C-2, 17), 25.6 (C-8, 11), 27.2 (C-5, 14), 29.4 (C-4, 15), 31.6 (C-3, 16), 127.7 (C-7, 12), 128.3 (C-9, 10), 130.4 (C-6, 13); Z6,Z9,Z12-19:H, 14.07, 14.10 (C-1, 19), 22.6, 22.7 (C-2, 18), 25.7 (× 2, C-8, 11), 27.25, 27.29 (C-5, 14), 29.0, 29.4, 29.7 (C-4, 15, 16), 31.6 (C-3), 31.8 (C-17), 127.7 (× 2, C-7, 12), 128.30 (× 2, C-9, 10), 130.4 (× 2, C-6, 13); Z6,Z9,Z12-20:H, 14.08, 14.12 (C-1, 20), 22.6, 22.7 (C-2, 19), 25.6 (×2, C-8, 11), 27.2, 27.3 (C-5, 14), 29.2, 29.3, 29.4, 29.7 (C-4, 15, 16, 17), 31.5 (C-3), 31.9 (C-18), 127.7 (× 2, C-7, 12), 128.27, 128.29 (C-9, 10), 130.4 (× 2, C-6, 13).

(3Z,6Z,9Z,12Z)-3,6,9,12-Tetraenes (Scheme B) Z3,Z6,Z9,Z12-17:H was synthesized starting from commercialized (*Z*)-3-octen-1-ol (**6a**). By oxidation with 1-hydroxy-1,2-benziodoxol-3(1*H*)-one 1-oxide (IBX), the alcohol was converted into the corresponding β,γ-unsaturated aldehyde with (*Z*)-configuration (**7a**). We used Dess-Martin oxidation with periodinate [1,1,1-tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3(1*H*)-one], but IBX was sufficient for the oxidation. NMR data for **7a**, which were differentiated easily from those of the corresponding (*E*)-isomer (Yamakawa et al., 2009), indicated high purity. Without any purification, the aldehyde was coupled with an ylide (**8**), which was prepared from the phosphonium salt of an iodide derived from (3*Z*,6*Z*)-3,6-nonadien-1-ol, to give the objective C_{17} tetraene. Z3,Z6,Z9,Z12-18:H was synthesized *via* (*Z*)-3-nonenal (**7b**), which was prepared by oxidation of commercially available (*Z*)-3-nonen-1-ol (**6b**). Z3,Z6,Z9,Z12-19:H was synthesized starting from 3-butyn-1-ol (**4**). After protection of the alcohol as THP ether, 1-bromohexane was coupled with it to yield 3-octynyl compound (**5c**). Partial hydrogenation with Pd-$CaCO_3$-Pb and deprotection of **5c** gave (*Z*)-3-decen-1-ol (**6c**). The

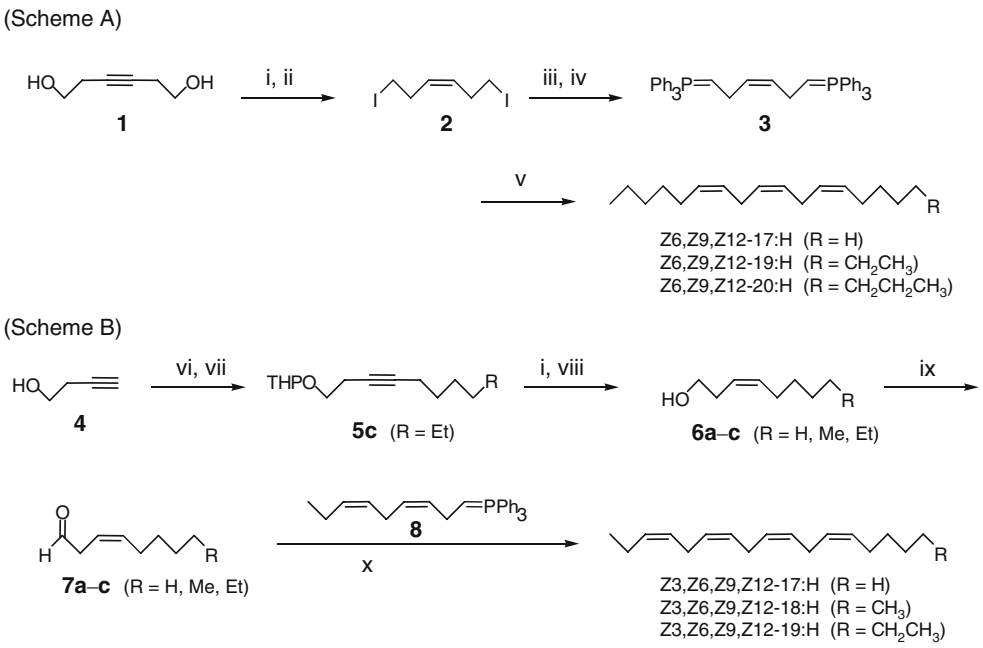


Fig. 1 Synthetic schemes for C_{17}–C_{20} (6*Z*,9*Z*,12*Z*)-6,9,12-trienes (Scheme A) and (3*Z*,6*Z*3,9*Z*,12*Z*)-3,6,9,12-tetraenes (Scheme B). Reagents: (i) H_2/Pd-$CaCO_3$-Pb/MeOH; (ii) I_2-PPh_3/imidazole/THF; (iii) PPh_3/benzene; (iv) $NaN(SiMe_3)_3$/THF; (v) $CH_3(CH_2)_4CHO$ + $CH_3(CH_2)_nCHO$ (*n*=3–6); (vi) DHP/PTSA; (vii) (1) BuLi/THF, (2) $CH_3(CH_2)_5Br$/HMPA; (viii) PTSA/EtOH; (ix) IBX/DMSO; (x) ylide **8**/THF

alcohol was oxidized to (*Z*)-3-decenal (**7c**), which was coupled with the ylide (**8**) to yield the C_{19} tetraene. ^{1}H NMR δ ppm: 0.88 (3H, d, *J*=7 Hz), 0.98 (3H, t, *J* = 7.5 Hz), ~1.3 (4H–8H for C_{17}–C_{19} tetraenes, m), 2.06 (4H, m), 2.82 (6H, m), 5.37 (8H, m). ^{13}C NMR δ ppm: Z3,Z6,Z9,Z12-17:H, 14.0 (C-17), 14.3 (C-1), 20.6 (C-2), 22.4 (C-16), 25.55, 25.63 and 25.64 (C-5, 8, 11), 27.0 (C-14), 31.8 (C-15), 127.1 (C-4), 127.6 (C-12), 127.96, 128.00, 128.50, and 128.52 (C-6, 7, 9, 10), 130.4 (C-13), and 132.0 (C-3); Z3,Z6,Z9,Z12-18:H, 14.1 (C-18), 14.3 (C-1), 20.6 (C-2), 22.7 (C-17), 25.56, 25.63 and 25.66 (C-5, 8, 11), 27.3 (C-14), 29.3 (C-15), 31.9 (C-16), 127.1 (C-4), 127.6 (C-12), 127.96, 128.01, 128.50, and 128.52 (C-6, 7, 9, 10), 130.5 (C-13), and 132.0 (C-3); Z3,Z6,Z9,Z12-19:H, 14.1 (C-19), 14.3 (C-1), 20.6 (C-2), 22.7 (C-18), 25.55, 25.63 and 25.66 (C-5, 8, 11), 27.3 (C-14), 29.0 and 29.7 (C-15, 16), 31.8 (C-17), 127.1 (C-4), 127.6 (C-12), 127.95, 128.01, 128.49, and 128.52 (C-6, 7, 9, 10), 130.5 (C-13), 132.0 (C-3); Z3,Z6,Z9,Z12-20:H, 14.1 (C-20), 14.3 (C-1), 20.6 (C-2), 22.7 (C-19), 25.56, 25.63 and 25.66 (C-5, 8, 11), 27.3 (C-14), 29.25, 29.31 and 29.7 (C-15, 16, 17), 31.9 (C-18), 127.1 (C-4), 127.6 (C-12), 127.95, 128.00, 128.49, and 128.52 (C-6, 7, 9, 10), 130.5 (C-13), 132.0 (C-3).

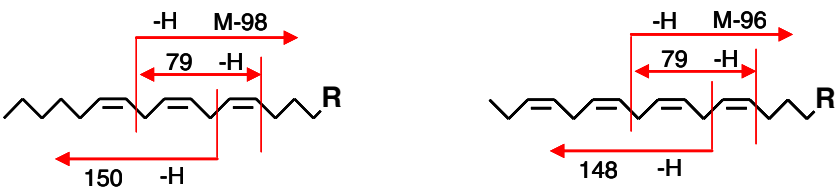
Field Trapping of Male Moths Rubber septa (white rubber, OD 8 mm; Sigma-Aldrich, St. Louis, MO, USA) were used as dispensers, and synthetic components (>98% purities on GC-MS analyses) dissolved in hexane (100 μl) were applied to them. Each lure was placed at the center of a sticky board trap (a 30×27 cm bottom plate with a roof; Sumitomo Chem. Co., Tokyo, Japan), which was set separately from the others by at least 10 m at about 1.5 m above the ground. Random screening tests for the synthetic polyenes were carried out in a mixed forest at Tama Forest Science Park in Hachioji in western Tokyo (24.42°N, 123.78°E), which includes many deciduous and broad-leaved evergreen trees, as well as many conifers, in 2008 and 2009. Three traps were used for each lure, and three other traps, including septa treated with only hexane (100 μl), were tested as a control to confirm specific attraction of male moths of two Geometrinae species, *Idiochlora ussuriaria* and *Jodis lactearia*. The lures were renewed every 3 mo, and the captured insects were examined twice a month from March to October. Synthetic pheromone lures targeting the *Hemithea tritonaria* males were evaluated at the forest in the Iriomote Islands, where the females were collected with a black light, in 2010 in a similar manner as for the random tests.

Table 1 Relative intensities of M^+ and some fragment ions in EI mass spectra of synthetic 6,9,12-trienes, 3,6,9,12-tetraenes, and the positional isomers

		Relative intensity (%) of fragment ions at indicated *m/z*									M^+	
		m/z	79[a-c]	80[a]	108[b]	110[c]	150[a]	163[c]	M–98[a]	M–56[b]	*m/z*	(%)
6,9,12-Triene	Z6,Z9,Z12-17:H		100	83	3	4	23	2	28	1	234	1
	Z6,Z9,Z12-18:H		100	91	6	4	72	4	72	0	248	1
	Z6,Z9,Z12-19:H		99	100	4	9	31	2	28	0	262	1
	Z6,Z9,Z12-20:H		94	100	6	6	44	3	38	2	276	1
3,6,9-Triene	Z3,Z6,Z9-19:H		100	59	65	3	1	2	0	24	262	2
6,9,11-Triene	Z6,Z9,E11-19:H		100	68	7	20	4	19	11	3	262	13
		m/z	79[d-f]	91[e]	106[e]	108[f]	148[d]	M–96[d]	M–82[f]	M–54[e]	*m/z*	(%)
3,6,9,12-Tetraene	Z3,Z6,Z9,Z12-17:H		100	45	7	14	5	11	3	3	232	0.2
	Z3,Z6,Z9,Z12-18:H		100	45	7	16	4	9	2	2	246	0.2
	Z3,Z6,Z9,Z12-19:H		100	45	8	16	5	8	1	1	260	0.2
	Z3,Z6,Z9,Z12-20:H		100	42	8	16	4	7	1	1	274	0.2
1,3,6,9-Tetraene	1,Z3,Z6,Z9-19:H		100	93	28	3	3	0	0	15	260	1
3,6,9,11-Tetraene	Z3,Z6,Z9,E11-19:H		100	30	4	69	0	0	6	0	260	2

GC-MS of each compound was measured by EI ionization operated at 70 eV

[a-f] The diagnostic fragment ions for 6,9,12-treines (a), 3,6,9-trienes (b), 6,9,11-trienes (c), 3,6,9,12-tetraenes (d), 1,3,6,9-tetraenes (e), 3,6,9,11-tetraenes (f). The spectra data of 3,6,9- and 6,9,11-trienes and 1,3,6,9- and 3,6,9,11-tetraenes have been reported in our precious paper (Yamamoto et al., 2008) and other publications (Bestmann et al., 1982; Wong et al., 1984; Frérot et al., 1993; Millar, 2000). The following structures are proposals for the formation of diagnostic fragment ions of 6,9,12-trienes and 3,6,9,12-tetraenes



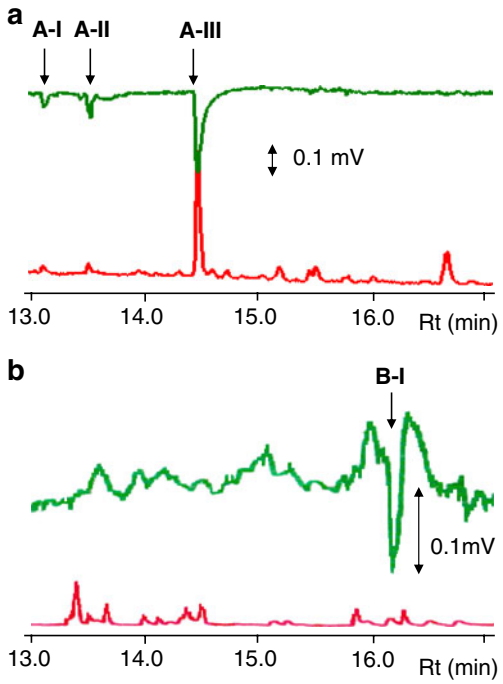


Fig. 2 GC-EAD analysis of the sex pheromone components of two Geometrinae species. **a**) Analysis of a crude pheromone extract of *Pamphlebia rubrolimbraria rubrolimbraria* (0.5 FE), indicating three EAG-active components [**A-I** (Z6,Z9-17:H), Rt 13.1 min; **A-II** (Z6, Z9,Z12-17:H), Rt 13.5 min; **A-III** (Z6,Z9,Z12-18:H), Rt 14.5 min]; and **b**) analysis of a crude pheromone extract of *Maxates versicauda microptera* (0.5 FE), indicating one EAG-active component [**B-I** (Z6, Z9,Z12-20:H), Rt 16.2 min]

Results

Characterization of Synthetic 6,9,12-Trienes and 3,6,9,12-Tetraenes The chemical structures of the synthetic polyenes were confirmed by NMR analysis. ^{1}H NMR analysis of each (6*Z*,9*Z*,12*Z*)-6,9,12-triene showed signals of six olefinic protons overlapped at δ 5.37 ppm. The ^{13}C NMR spectrum of Z6,Z9,Z12-18:H showed only nine signals because of its symmetric structure, and the allylic carbon signal at δ 27.2 ppm and double-allylic carbon signal at δ 25.6 ppm indicated the (*Z*)-configuration of three double bonds. Since the structures of other (6*Z*,9*Z*,12*Z*)-6,9,12-trienes are almost symmetric, overlapped signals of six olefinic carbons were recorded at similar chemical shifts of Z6,Z9,Z12-18:H. ^{1}H NMR analysis of each (3*Z*,6*Z*,9*Z*,12*Z*)-3,6,9,12-tetraene showed signals of eight olefinic protons also overlapped at δ 5.37 ppm. The ^{13}C NMR spectrum of each tetraene included eight olefinic carbon signals, which were interspersed from δ 127.1 ppm to δ 132.0 ppm with a similar pattern as that of (3*Z*,6*Z*,9*Z*)-3,6,9-trienes (Ando et al., 1993). The chemical shifts of three double-allylic carbon signals that appeared around δ 25.6 ppm indicated (*Z*)-configuration of four double bonds.

Table 1 shows the relative intensities of M^+ and some fragment ions in the EI mass spectra. While Z6,Z9,Z12-19:H and Z6,Z9,Z12-20:H produced a base peak at *m/z* 80, the 6,9,12-trienes universally showed $[H(CH=CH)_3]^+$ with a strong intensity at *m/z* 79. The spectra of four 6,9,12-trienes indicated that fragment ions at *m/z* 79, 80, 150, and M–98 are diagnostic. In the spectra of the 6,9,12-trienes, the relative intensities of ions at *m/z* 108 and M–56, which are diagnostic for 3,6,9-trienes, and ions at *m/z* 110 and 163, which are diagnostic for 6,9,11-trienes, are weak. Therefore, the 6,9,12-tienes are distinguished easily from the positional isomers. The mass spectra of four 3,6,9,12-tetraenes indicated diagnostic ions at *m/z* 79, 148, and M–96, but the intensities of latter two ions were not high. In the spectra of the 3,6,9,12-tetraenes, the relative intensities of ions at *m/z* 106 and M–54, which are diagnostic for

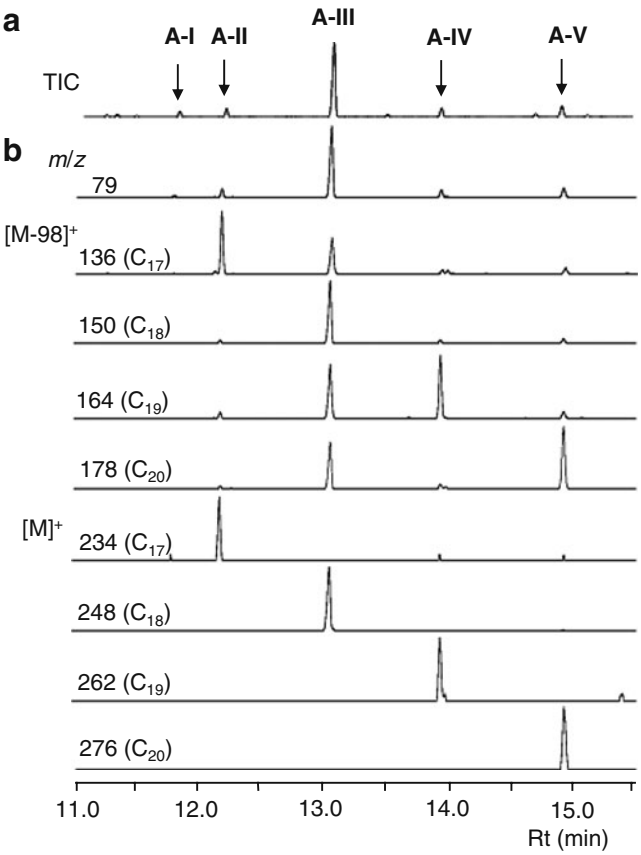


Fig. 3 GC-MS analysis of a crude pheromone extract of *Pamphlebia rubrolimbraria rubrolimbraria* (0.5 FE). **a**) Total ion chromatogram (TIC); and **b**) mass chromatograms of the ion at *m/z* 79 (a base peak) and other diagnostic ions of C_{17}–C_{20} 6,9,12-trienes, i.e., $[M–98]^+$ at *m/z* 136, 150, 164, and 178, and M^+ at *m/z* 234, 248, 262, and 276. The chromatograms show elution of the following four trienes: **A-II** (Z6,Z9,Z12-17:H), Rt 12.17 min; **A-III** (Z6,Z9,Z12-18:H), Rt 13.06 min; **A-IV** (Z6,Z9,Z12-19:H), Rt 13.91 min; and **A-V** (Z6,Z9, Z12-20:H), Rt 14.92 min

Table 2 Field attraction of male emerald moths by synthetic polyenes at a mixed forest in Tokyo

Lure	Numbers of captured males of Geometrinae species (A–C) [a]								
	March	Apr.	May	June	July	Aug.	Sept.	Oct.	Total
Z6,Z9,Z12-17:H	0	0	0	A: 5	A: 35	A: 17	A: 21	0	A: 78
Z6,Z9,Z12-20:H	0	0	0	B: 4	0	0	0	0	B: 4
Z3,Z6,Z9,Z12-20:H	0	C: 135	C: 63	0	0	0	0	0	C: 198
Others [b]	0	0	0	0	0	0	0	0	0

Three traps were used for each lure

[a] A, *Idiochlora ussuriaria*; B, Gen. et sp.; C, *Jodis lactearia*

[b] Control and other 6,9,12-trienes and 3,6,9,12-tetraenes with a C_{17}–C_{20} chain

1,3,6,9-tetraenes, and ions at *m/z* 108 and M–82, which are diagnostic for 3,6,9,11-tetraenes, are very weak. The 3,6,9,12-tetraenes are distinguished easily from these positional isomers.

Analysis of Pheromone Gland Extracts from Two Geometrinae Species A GC-EAD analysis of a pheromone gland extract (0.5 female equivalent, FE) from *P. r. rubrolimbraria* recorded three EAG-active components (**A-I**, **A-II**, and **A-III**) as shown in Fig. 2a. While the contents of **A-I** and **A-II** were low and the activities were weak, the antennal responses were reproducibly observed (N=3). The extract (0.5 FE) was analyzed by GC-MS to reveal the structures. Figure 3 shows mass chromatograms, which monitor M^+ of C_{17}–C_{20} 6,9,12-trienes (*m/z* 234, 248, 262, and 276) and their diagnostic fragment ions at *m/z* M–98 (*m/z* 136, 150, 164, and 178) and *m/z* 79. In addition to the identification of Z6,Z9,Z12-17:H for **A-II** and Z6,Z9,Z12-18:H for **A-III**, this analysis also indicates inclusion of two other trienyl components, Z6,Z9,Z12-19:H (**A-IV**) and Z6,Z9,Z12-20:H (**A-V**). The Rt values and mass spectra of **A-II**–**A-V** coincided with those of the synthetic 6,9,12-trienes. The GC-MS data of **A-I** (Rt 11.80 min), which showed M^+ at *m/z* 236 and characteristic fragment ions at *m/z* 67, 81, and 110, coincided with those of C_{17} 6,9-diene, i.e., Z6,Z9-17:H. The total ion chromatogram (TIC) indicated the mixing ratio of **A-I** – **A-V** to be about 5:10:100:10:15. On the other hand, a GC-EAD analysis of a pheromone gland extract (0.5 FE) from *M. v. microptera* recorded only one EAG-active component (**B-I**), as shown in Fig. 2b. The GC-MS analysis revealed it to be Z6,Z9, Z12-20:H, while the mass chromatograms did not suggest inclusion of other unsaturated hydrocarbons.

Attraction of Geometrinae Males by the Polyenes To obtain information about sex pheromones of the species inhabiting the main island of Japan, each of the synthetic 6,9,12-trienes and 3,6,9,12-tetraenes with a C_{17}–C_{20} chain was evaluated as a single component lure in the suburb of Tokyo. To date, new male attraction of two Geometrinae species has been found as follows: *Idiochlora ussuriaria* by Z6,Z9,Z12-17:H and *Jodis lactearia* by Z3,Z6,Z9,Z12-20:H. Table 2 shows the numbers of captured males, which were totaled each month. *Idiochlora ussuriaria* is a defoliator in a chestnut orchard and tea garden, and is recognized as a pest insect in Japan. The males of each species were attracted specifically by one polyene. Neither traps for control nor traps baited with other compounds captured males of these species. Although the attraction was uncertain because of how few males were trapped, one

Table 3 GC-MS data of authentic farnesenes and the pheromone component $\mathbf{X_1}$ produced by *H. tritonaria* females

Each compound was chromatographed on a DB-23 capillary column (0.25 mm ID×30 m) under programmed temperature (50°C for 2 min, 10°C/min to 160°C, and 4°C/min to 220°C), and the mass spectrum was measured by EI ionization operated at 70 eV

	Rt (min)	Relative intensity (%) of M^+ and fragment ions at indicated *m/z*								
		69	93	107	119	123	133	147	161	204 (M)
β-farnesene										
(*Z*)-Isomer	10.31	100	63	10	10	2	31	3	16	3
(*E*)-Isomer	10.63	100	64	10	9	2	30	3	15	4
α-farnesene										
(3*Z*,6*E*)-Isomer	11.15	47	100	38	89	6	6	2	5	1
(3*E*,6*Z*)-Isomer	11.23	63	100	48	50	8	23	9	12	4
(3*E*,6*E*)-Isomer	11.42	50	100	48	42	36	6	2	5	1
Comp. $\mathbf{X_1}$	11.42	49	100	49	42	38	6	3	6	1

more Geometrinae species (Gen. et sp.) was captured using Z6,Z9,Z12-20:H. In this field screening test, the polyenes attracted no male moths belonging to other geometrid subfamilies or other lepidopteran families.

Identification of (3E,6E)-α-Farnesene In addition to Z6,Z9,Z12-18:H, the pheromone extract from *H. tritonaria* females included an unknown EAG-active substance, which was expressed as component $\mathbf{X_1}$ in our previous report (Yamakawa et al., 2009). The GC-MS analysis showed a short Rt, and M^+ at *m/z* 204, suggesting a C_{15} tetraene with a structure related to Z6,Z9,Z12-18:H. The base peak at *m/z* 93, however, cast doubt upon this possibility. In fact, synthetic Z3,Z6,Z9,Z12-15:H showed the same base peak at *m/z* 79 as the other 3,6,9,12-tetraenes did and was eliminated as a candidate. Eventually, analysis with a mass spectral database (NIST 98) indicated $\mathbf{X_1}$ to be α-farnesene. Direct comparison of the GC-MS data of $\mathbf{X_1}$ and the authentic standards of farnesene confirmed the identification, as shown in Table 3. Since the (3*Z*,6*E*)- and (3*E*,6*Z*)-isomers eluted faster than (3*E*,6*E*)-α-farnesene from the polar GC column used in this analysis, the Rt value of the (3*Z*,6*Z*)-isomer might be the smallest of four geometrical isomers of α-farnesene. GC-MS data of $\mathbf{X_1}$ coincided with those of (3*E*,6*E*)-α-farnesene. Although the authentic (3*Z*,6*Z*)-isomer was not available for a direct comparison, the (3*E*,6*E*)-configuration for $\mathbf{X_1}$ seems most likely.

Field Evaluation of (3E,6E)-α-Farnesene The activities of synthetic lures baited with a mixture of Z6,Z9,Z12-18:H and (3*E*,6*E*)-α-farnesene in a ratio of 10:0, 8:2, 5:5, 2:8, and 0:10 were examined in the Iriomote Islands. While the triene unmixed with farnesene captured a few males of *H. tritonaria*, as in our previous field test (Yamakawa et al., 2009), the number of captured males was significantly higher with the addition of farnesene (Fig. 4).

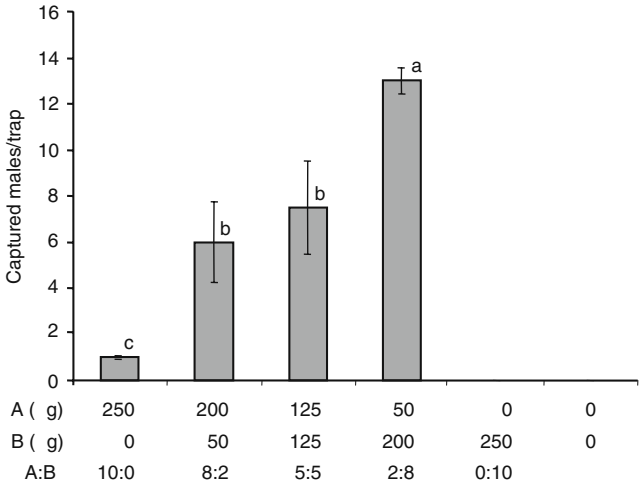


Fig. 4 Field evaluation of Z6,Z9,Z12-18:H (A) mixed with (3*E*,6*E*)-α-farnesene (B) for attraction of *Hemithea tritonaria* males. Tested at the Iriomote Islands from 1 July to 6 August 2009 using three traps for each lure. The bar indicates the means±standard errors (N=3). Columns with different lowercase letters are significantly different at P<0.05 by the Tukey-Kramer test

Discussion

As in a previous study of *Hemithea tritonaria* and *Thalassodes immissaria intaminata* (Yamakawa et al., 2009), we found novel 6,9,12-trienes in pheromone gland extracts from the females of *Pamphlebia rubrolimbraria rubrolimbraria* and *Maxates versicauda microptera* inhabiting a subtropical Japanese island (Figs. 2 and 3). Furthermore, field evaluation of the synthetic 6,9,12-trienes and 3,6,9,12-tetraenes revealed new instances of male attraction of other emerald moth species, *Idiochlora ussuriaria* and *Jodis lactearia*, on the main island (Table 2). Sex pheromones have not been investigated with any other Geometrinae species, and information about their pheromones is limited. These results, however, indicate that the double bond at a 12-position is characteristic of the sex pheromones secreted by some emerald moth species. Although many species of Geometrinae are defoliators, some agricultural pests, such as *I. ussuriaria*, belong to this taxonomic group.

As other Type II pheromones, 6,9,12-trienes and 3,6,9,12-tetraenes are expected to be produced starting from linoleic and linolenic acids, respectively, via biochemical reactions, such as chain elongation and decarboxylation. However, the key biosynthetic step is additional desaturation at the ω12-position of the dietary acids or the corresponding long-chain acids. The species in highly evolved lepidopteran groups mainly communicate by utilizing 6,9-dienes, 3,6,9-trienes, and their epoxides, which are derived from the acids without further modification of the polyene system (Ando et al., 2008). The newly identified 4,6,9- and 6,9,11-trienes, 1,3,6,9- and 3,6,9,11-tetraenes, and their epoxides (Ando et al., 2004; Ando, 2010) reveal additional pheromone diversity in this group of moths. These polyenes include an additional double bond at a conjugated position. The polyenes, which are unsaturated at the unique 12-position without a conjugated system, have not been identified from any species in Macrolepidoptera except for the emerald moths. Recently, 3,6,9,12,15-tricosapentaene and pentacosapentaene were discovered from three species in Pyralidae (Leal et al., 2005; Millar et al., 2005), many other species of which produce solely Type I pheromones. The pentaenes may be biosynthesized from linolenic acid by two additional desaturation steps, such as the biosynthesis of arachidonic acid from linolic acid (desaturation at the ω12- and ω15-positions). The Geometrinae pheromones found in our study are new natural products, which can be derived from

linolic or linolenic acids by only one desaturation at the ω12-position. It would be interesting to determine whether or not this limited desaturation is universally characteristic of the pheromones of many Geometrinae species.

It is noteworthy that (3*E*,6*E*)-α-farnesene was found in a pheromone extract of an emerald moth, *H. tritonaria*. The male antennae responded to synthetic (3*E*,6*E*)-α-farnesene, and inclusion of this sesquiterpene synergized the attraction of males to Z6,Z9,Z12-18:H. α-Farnesene was isolated from natural coating of apples, and Dufour's gland in ants in the 1960s. Since then, the compound has been identified from many plants and insects (El-Sayed, 2010). α-Farnesene from plants wounded by herbivores is thought to play a role in plant defense by attracting predators and parasitoids (Van den Boom et al., 2004). In the case of Lepidoptera, α-farnesene occurs in androconial organs in males of a swift moth (Schulz et al., 1990) and Danainae butterflies (Schulz et al., 1993), and larvae of some Papilionidae butterflies secrete β-farnesene from osmeteria to defend the attacks of predatory enemies, but this is the first instance in which farnesene is part of a female moth sex pheromone. *In vivo* experiments with ^{13}C-labeled precursors revealed that β-farnesene was biosynthesized by the usual terpenoid pathway in the organ (Honda, 1990), suggesting the possibility of *de novo* synthesis of α-farnesene in the emerald moth. Females of many species, including moths (Yan et al., 2003; Binder et al., 1995), recognize α-farnesene as a chemical cue to find their host plants, indicating that the antennae of females evolved receptors specific to this compound. The *H. tritonaria* males evidently have a similar perception system to smell α-farnesene from virgin conspecific females. How and why emerald moth incorporate this commonly occurring sesquiterpene in sex pheromone is mysterious. Further investigation with other species of Geometrinae should be developed to understand the specificity of this unique combination of compounds from two different biochemical pathways, and to determine if β-farnesene or other geometrical isomers of α-farnesene are pheromone components in other species.

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Determination of the Relative and Absolute Configurations of the Female-produced Sex Pheromone of the Cerambycid Beetle *Prionus californicus*

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Abstract We previously identified the basic structure of the female-produced sex attractant pheromone of the cerambycid beetle, *Prionus californicus* Motschulsky (Cerambycidae: Prioninae), as 3,5-dimethyldodecanoic acid. A synthesized mixture of the four stereoisomers of 3,5-dimethyldodecanoic acid was highly attractive to male beetles. Here, we describe stereoselective syntheses of three of the four possible stereoisomers, and the results of laboratory and field bioassays showing that male beetles are attracted specifically to (3*R*,5*S*)-3,5-dimethyldodecanoic acid, but not to its enantiomer, (3*S*,5*R*)-3,5-dimethyldodecanoic acid, indicating that the (3*R*,5*S*)-enantiomer is the active pheromone component. The diastereomeric (3*R*,5*R*)- and (3*S*,5*S*)-enantiomers were excluded from consideration because their gas chromatographic retention times were different from that of the insect-produced compound. The mixture of the four stereoisomers of 3,5-dimethyldodecanoic acid was as attractive to male *P. californicus* as the (3*R*,5*S*)-enantiomer, indicating that none of the other three stereoisomers inhibited responses to the active enantiomer. Beetles responded to as little as 10 ng and 10 μg of synthetic 3,5-dimethyldodecanoic acid in laboratory and field studies, respectively. Field studies indicated that capture rate did not increase with dosages of 3,5-dimethyldodecanoic acid greater than 100 μg. In field bioassays, males of a congeneric species, *P. lecontei* Lameere, were captured in southern California but not in Idaho.

Key Words Cerambycidae · Prioninae · Sex pheromone · (3*R*,5*S*)-dimethyldodecanoic acid

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Introduction

Volatile male-produced sex or aggregation pheromones have been reported for an increasing number of cerambycid beetle species (Silk et al., 2007; Lacey et al., 2009; Millar et al., 2009, and references therein; Ray et al., 2009a, b), most of which are members of the subfamilies Aseminae and Cerambycinae (Monné and Hovore, 2005). Female-produced pheromones also have been identified for two species that formerly were placed in the Cerambycidae, *Migdolus fryanus* Westwood and *Verperus xatarti* Dufour (Leal et al., 1994; Boyer et al., 1997), but whose family-level taxonomy is uncertain (Napp, 1994; Bense, 1995; Dong and Yang, 2003). Many cerambycid species in the subfamily Prioninae have morphological and behavioral traits that are associated with production of pheromones by females in other types of insects (Thornhill and Alcock, 1983; Gemeno and Schal, 2003), including more elaborate

antennal structures in males than females, and a more sedentary nature in females than males (Summerland, 1932; Benham and Farrar, 1976; Santos Ferreira, 1980; Hovore, 1981). Moreover, males of some prionine species display behaviors indicative of responses to volatile pheromones produced by females (Rotrou, 1936; Edwards, 1961; Benham and Farrar, 1976; Gwynne and Hostetler, 1978).

The prionine species *Prionus californicus* Motschulsky, is broadly distributed in western North America and is an economically important pest of hop, *Humulus lupulus* L. (Urticales: Cannabaceae), in the northwestern United States (Bishop et al., 1984). Adult *P. californicus* are active during summer and early fall, and are crepuscular (Linsley, 1962). They are among the largest cerambycids in North America, ranging in body length from 2.5 to 4.5 cm (volumes indexed in Linsley and Chemsak, 1997). Adult females oviposit in soil near the base of living woody plants; larvae feed on roots and rhizomes, requiring 3–5 yr to complete development (Linsley, 1962).

We previously reported that female *P. californicus* produce a volatile sex pheromone associated with an eversible structure on the ovipositor, and that males are strongly attracted to this pheromone (Cervantes et al., 2006; Barbour et al., 2007). The basic structure of the pheromone was identified as 3,5-dimethyldodecanoic acid by gas chromatographic (GC) retention time and mass spectral matches with those of one of the two peaks in a synthetic standard composed of an approximately equal mixture of all four stereoisomers of this compound (Rodstein et al., 2009). In the work reported here, we determined that the biologically active stereoisomer is (3*R*,5*S*)-dimethyldodecanoic acid and confirmed the activity of the synthetic pheromone in laboratory and field bioassays. We also showed that the mixture of stereoisomers is as active as the insect-produced enantiomer, indicating that the unnatural stereoisomers do not antagonize attraction to the natural enantiomer.

Methods and Materials

Source of Insects Larvae of *P. californicus* were collected in fall of 2007 from commercial hop yards in southwestern Idaho and reared to adulthood in a field plot at the Southwest Idaho Research and Extension Center (Canyon Co., ID, USA). Larvae were reared individually in 19-l plastic buckets buried in the ground to within ~10 cm of their tops. Each bucket had two 0.75 cm holes in the bottom for drainage, and buckets were filled with soil to ~10 cm from the top. We collected late stage larvae (5–7.5 cm long) from uprooted hop crowns, and buried each larva ~10 cm deep in the soil in a bucket, with a section of fresh hop rhizome for food.

We cut back hop plants growing from rhizomes in the buckets in early spring 2008, and we fitted buckets with lids that had the centers replaced with aluminum screen. Each bucket was drip-irrigated (8 l/hr) for 10 min every other day. Adult beetles emerged in buckets in July 2008 and were collected and held individually in the laboratory in ventilated glass jars (~500–1000 ml; 22°C, 16:8 hr L:D). Males and females were unmated and held in separate growth chambers. Beetles used in bioassays were 1–14-d-old, vigorous, and apparently healthy.

Syntheses of Stereoisomers of 3,5-Dimethyldodecanoic Acid Tetrahydrofuran was dried and purified by distillation from sodium-benzophenone ketyl under argon. Solutions of crude products were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure, unless specified otherwise. Crude products were purified by flash or vacuum flash chromatography on 230–400-mesh silica gel. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian INOVA-400 spectrometer (Palo Alto, CA, USA) (400 and 100.5 MHz, respectively), as $CDCl_3$ solutions. Integer resolution mass spectra were obtained with a Hewlett-Packard (HP) 6890 GC (Avondale, PA) interfaced to an HP 5973 mass spectrometer (MS), in EI mode (70 eV) with helium carrier gas. The GC was equipped with a DB5-MS column (25 m×0.20 mm ID × 0.25 μ film). Resolutions of enantiomers were attempted on a Cyclodex-B column (30 m×0.3 mm ID, J&W Scientific, Folsom, CA, USA) using isothermal conditions or temperature programs of 2–4°C per min. Optical rotations were measured in CH_2Cl_2 on a Perkin Elmer 241 polarimeter (Na and Hg lamps) (Perkin Elmer, Waltham, MA, USA) in a 1 ml water-jacketed cell (10 cm path length) at 24°C. All rotations were averaged over three or four measurements. IR spectra were taken as films on NaCl plates on a Bruker Equinox 55 FTIR instrument (Freemont, CA, USA).

*Methyl (E)-2-Decenoate (**2**) (Fig. 1)* (*E*)-2-decenoic acid (**1**) (20.91 g, 123 mmol, Acros Organics, Morris Plains, NJ, USA) was dissolved in methanol (82 ml) in a dry flask under argon at room temperature. Acetyl chloride (1.74 ml, 25 mmol) was added carefully dropwise (vigorous reaction!), and the reaction stirred for 16 h and then quenched with aqueous sodium bicarbonate. The resulting mixture was extracted x 4 with ether. The combined ether extracts were washed with brine, dried, and concentrated. The resulting oil was Kugelrohr distilled (oven temp. ~87°C, 1 Torr) to yield 14.95 g (81 mmol, 66%) of methyl (*E*)-2-decenoate (**2**). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (t, *J*= 6.8 Hz, 3 H), 1.29 (m, 8 H), 1.46 (m, 2 H), 2.16–2.23 (m, 2 H), 3.73 (s, 3 H), 5.82 (d, *J*=15.6 Hz, 1 H), 6.98 (dt, *J*= 15.4, 7.0 Hz, 1 H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 14.2, 22.8, 28.2, 29.3 (2C), 31.9, 32.4, 51.5, 120.9, 150.0, 167.4.

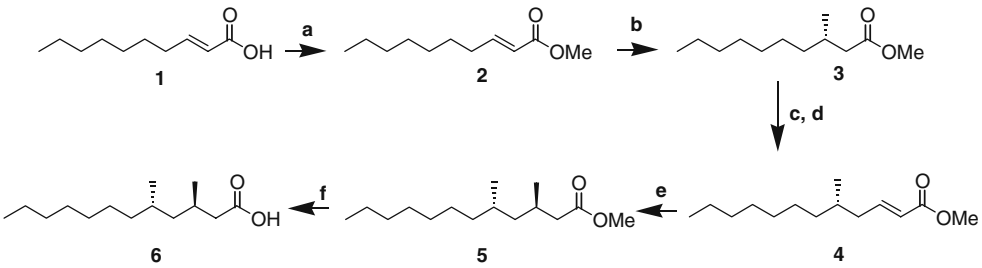


Fig. 1 Synthesis of (*3R,5S*)-(+)-3,5-dimethyldodecanoic acid. a) acetyl chloride, MeOH; b) MeMgBr, CuI, (*S*)-Tol-BINAP, *t*-butylmethyl ether; c) DIBAL-H, hexane; d) methyl (triphenylphosphoranylidene) acetate, THF; e) MeMgBr, CuI, (*R*)-Tol-BINAP, *t*-butylmethyl ether; f) KOH, MeOH/H_2O

MS: *m/z* (%): 153 (M^+ - 31, 3), 141 (3), 110 (20), 96 (17), 87 (81), 69 (40), 55 (86). IR (film): 2953, 2928, 2856, 1728, 1658, 1460, 1436, 1378, 1313, 1270, 1197, 1170, 1127, 1041, 979, 921, 854, 720 cm^{-1}.

*Methyl (S)-(-)-3-Methyldecanoate (***3***)* (*S*)-(-)-2,2′-Bis(di-*p*-tolylphosphino)-1,1′-binaphthyl (= (*S*)-Tol-BINAP, 0.45 g, 0.66 mmol; Alfa Aesar, Ward Hill, MA, USA) and CuI (84 mg, 0.44 mmol) were added to a dry three-neck flask charged with CH_2Cl_2 (55 ml) under argon. After stirring 30 min, the CH_2Cl_2 was removed *in vacuo* and replaced with *t*-BuOMe (27 ml), and the mixture stirred for 30 min. The *t*-BuOMe was removed *in vacuo* and the resulting neon green-yellow solid diluted with a second portion of *t*-BuOMe (88 ml). The mixture then was cooled to −20°C and MeMgBr (38.3 ml, 2.4 M solution in Et_2O, 92 mmol) added dropwise. After stirring for 20 min, a solution of methyl (*E*)-2-decenoate **2** (4.07 g, 22.1 mmol) in *t*-BuOMe (27 ml) was added via syringe pump over 4.5 h, maintaining the temperature at or slightly below −20°C. After stirring at −20°C for another 2 h, the reaction mixture was quenched with MeOH (10 ml), followed by saturated NH_4Cl solution. The mixture was extracted with Et_2O (4×75 ml) and the combined organic extracts were washed with brine, dried, and concentrated. The residue was purified by flash chromatography (hexane/EtOAc 97:3), then Kugelrohr distilled (oven temp. ~92°C, 1 Torr) yielding 2.39 g (11.9 mmol, 53%) of methyl (*S*)-(-)-3-methyldecanoate (**3**). $[\alpha]_D^{20}$=−4.2° (c=2.03, CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, *J*=6.7 Hz, 3 H), 0.93 (d, *J*=6.6 Hz, 3 H), 1.26 (m, 12 H), 1.95 (m, 1 H), 2.09 (m, 1 H), 2.31 (dd, *J*=14.7, 6.0 Hz, 1 H), 3.67 (s, 3 H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 14.3, 19.9, 22.9, 27.1, 29.5, 29.9, 30.6, 32.1, 36.9, 41.9, 51.5, 174.0. MS: *m/z* (%): 200 (M^+, 1), 185 (1), 169 (3), 157 (3), 143 (2), 101 (39), 87 (5), 74 (100), 55 (16), 43 (38). IR (film) 2957, 2928, 2855, 1732, 1460, 1437, 1379, 1167 cm^{-1}.

*Methyl (2E,5S)-(-)-5-Methyldodec-2-enoate (***4***)* Methyl (*S*)-(-)-3-methyldecanoate (**3**) (1.79 g, 8.93 mmol) was dissolved in hexane (10 ml) in a dry flask under argon, and cooled to −78°C. Diisobutylaluminum hydride (DIBAL-H, 1 M in hexane, 9.8 ml, 9.8 mmol; Aldrich Chemical Co., Milwaukee, WI, USA) was added dropwise. The mixture was stirred for 1 hr, and then another 1 ml of DIBAL-H was added to reduce small amounts of residual starting material. After stirring another 30 min, MeOH (1.13 ml) was added dropwise, and the mixture stirred for 30 min during which time a white suspension formed. Methyl (triphenylphosphoranylidene) acetate (5.97 g, 17.9 mmol; Aldrich Chemical Co.) then was added at −78°C, followed by THF (45 ml). The reaction was allowed to warm to room temperature overnight and stirred for an additional 48 h. The mixture was diluted with Et_2O (20 ml) and 6 M HCl (40 ml), and stirred until two clear layers were observed. After separation of the organic layer, the aqueous phase was extracted x 3 with Et_2O, and the combined organic extracts were washed with saturated aqueous $NaHCO_3$ and brine, dried, and concentrated. The residue was taken up in hexane and filtered through a small plug of silica gel to remove the bulk of the triphenylphosphine oxide. The filtrate was concentrated and purified by flash chromatography (hexane/EtOAc 98:2 then 96:4). The product was purified further by Kugelrohr distillation (oven temp. ~110°C, 1 Torr) to yield methyl (2*E*,5*S*)-(-)-5-methyldodec-2-enoate (**4**) (1.01 g, 2.5 mmol, 50%). $[\alpha]_D^{20}$=−1.3° (c=1.99, CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, *J*= 6.4 Hz, 3 H), 0.89 (d, *J*=6.8, 3 H), 1.26 (m, 12 H), 1.60 (m, 1 H), 2.03 (m, 1 H), 2.20 (m, 1 H), 3.73 (s, 3 H), 5.81 (d, *J*= 15.6 Hz, 1 H), 6.95 (m, 1 H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 14.3, 19.8, 22.9, 27.2, 29.5, 30.0, 32.1, 32.7, 36.8, 39.9, 51.5, 122.1, 148.9, 167.3. MS: *m/z* (%): 195 (2, M^+-33), 155 (1), 142 (5), 100 (100), 85 (11), 71 (21), 57 (27), 43 (60). IR (film) 3054, 2956, 2927, 2855, 2306, 1718, 1655, 1437, 1378, 1322, 1265, 1195, 1143, 982, 909, 737 cm^{-1}.

*Methyl (3R,5S)-(+)-3,5-Dimethyldodecanoate (***5***)* (*R*)-Tol-BINAP (54 mg, 0.08 mmol) and CuI (10 mg, 0.053 mmol) were stirred in CH_2Cl_2 (10 ml) in a dry flask under argon. After 30 min, the solvent was removed *in vacuo* and replaced with *t*-BuOMe (10 ml), and the mixture stirred for 30 min. The *t*-BuOMe was removed *in vacuo*, and the resulting green-yellow solid was resuspended in *t*-BuOMe (10 ml). The mixture was cooled to −20°C and MeMgBr (5.4 ml, 2.5 M solution in Et_2O, 13.5 mmol) added dropwise. After stirring for 20 min, a solution of methyl (2*E*,5*S*)-(-)-5-methyldodec-2-enoate (**4**) (0.6 g, 2.6 mmol)

in *t*-BuOMe (3.2 ml) was added via syringe pump over 4.5 hr. After stirring at −20°C for another 2 hr, the reaction mixture was quenched with MeOH (3 ml), followed by saturated NH_4Cl solution. The mixture was extracted with Et_2O (4x), and the combined organic extracts were washed with brine, dried, and concentrated. The residue was purified by flash chromatography (hexane/EtOAc, 97:3), then Kugelrohr distillation (oven temp. ~100°C, 1 Torr) to yield 0.074 g (0.31 mmol, 12%) of methyl (*3R,5S*)-(+)-3,5-dimethyldodecanoate (**5**) (diastereomeric purity 90.7% by GC). $[\alpha]_D^{20}$=+16.0º (c=1.4, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.84 (t, *J*=6.8 Hz, 3 H), 0.89 (d, *J*=6.8 Hz, 3 H), 0.90 (d, *J*=6.4 Hz, 3 H), 1.12 (m, 2 H), 1.27 (m, 12 H), 1.46 (br s, 1 H), 2.08 (m, 2 H), 2.27 (dd, *J*=14.4, 6.0 Hz, 1 H), 3.67 (s, 3 H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 14.3, 19.5, 19.6, 22.9, 27.2, 28.0, 29.6, 30.1, 30.2, 32.1, 37.9, 42.6, 44.6, 51.5, 173.9. MS: *m/z* (%): 242 (M^+, 1), 227 (1), 211 (1), 185 (4), 169 (2), 157 (1), 143 (4), 125 (2), 115 (5), 101 (40), 83 (12), 74 (100), 55 (24), 43 (47). IR (film) 2957, 2927, 2854, 1733, 1461, 1436, 1379, 1191, 1007, 910 cm^{-1}.

*(3R,5S)-(+)-3,5-Dimethyldodecanoic Acid (**6**)* A mixture of methanol (1.1 ml), water (1.2 ml), KOH (0.32 g, 5.2 mmol), and methyl (3*R*,5*S*)-(+)-3,5-dimethyldodecanoate (**5**) (70 mg, 0.29 mmol) were stirred overnight at room temperature. The mixture was extracted with hexane, and the remaining aqueous layer acidified with 1M HCl and extracted three times with Et_2O. The combined ether layers were washed with brine, dried, and concentrated, yielding (3*R*,5*S*)-(+)-3,5-dimethyldodecanoic acid (**6**) (54.2 mg, 82%). $[\alpha]_D^{20}$=+18.1º (c=1.08, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (m, 6 H), 0.94 (d, *J*=6.4 Hz, 3 H), 1.14 (m, 2 H), 1.27 (m, 12 H), 1.46 (br s, 1 H), 2.06 (m, 1 H), 2.17 (dd, *J*=14.8, 8.0 Hz, 1 H), 2.31 (dd, *J*=14.8, 6.0 Hz, 1 H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 14.3, 19.5, 19.6, 22.9, 27.2, 27.8, 29.5, 30.1, 30.2, 32.1, 37.9, 42.4, 44.5, 179.1. MS: *m/z* (%): 228 (M^+, 1), 171 (8), 129 (14), 111 (11), 101 (13), 87 (74), 69 (42), 57 (57), 43 (100). IR (film) 2958, 2925, 2872, 2854, 1709, 1463, 1410, 1379, 1296, 1233, 1196, 937, 722 cm^{-1}.

*Methyl (R)-(+)-3-Methyldecanoate (**7**)* This compound was prepared in analogous fashion to compound **3** from methyl (*E*)-2-decenoate (**2**), using (*R*)-(+)-2,2′-bis(di-p-tolylphosphino)-1,1′-binaphthyl (= (*R*)-Tol-BINAP) as catalyst, yielding methyl (*R*)-(+)-3-methyldecanoate (**7**) in 49% isolated yield. $[\alpha]_D^{20}$=+4.8° (c=2.02, CH_2Cl_2). ^{1}H NMR, ^{13}C NMR, IR, and mass spectra matched those of the (*S*)-enantiomer (**3**).

*Methyl (2E,5R)-(+)-5-Methyldodec-2-enoate (**8**)* This compound was made in analogous fashion to compound **4** by using methyl (*R*)-(+)-3-methyldecanoate (**7**) instead of the methyl (*S*)-(-)-3-methyldecanoate (**3**) to afford the desired (*E*)-alkenoate in 27% yield after purification. A further 41% of a mixture of the (*E*)- and (*Z*)-isomers also was obtained. $[\alpha]_D^{20}$=+1.8º (c=2.06, CH_2Cl_2). The ^{1}H NMR, ^{13}C NMR, IR, and mass spectra agreed with those of the (*S*)-enantiomer **4**.

*Methyl (3S,5R)-(-)-3,5-Dimethyldodecanoate (**9**)* This enantiomer was made in analogous fashion to compound **5** in 23% isolated yield from methyl (2*E*,5*R*)-(+)-5-methyl-2-dodecenoate (**8**) (diastereoisomeric purity, 92.5%), using (*S*)-Tol-BINAP instead of (*R*)-Tol-BINAP as catalyst. $[\alpha]_D^{20}$=−18.2° (c=1.13, CH_2Cl_2). ^{1}H NMR, ^{13}C NMR, IR, and mass spectra matched those of the corresponding (3*R*,5*S*)-(+)-enantiomer (**5**).

*(3S,5R)-(-)-3,5-Dimethyldodecanoic Acid (**10**)* Methyl (3*S*,5*R*)-(-)-3,5-dimethyldodecanoate (**9**) was hydrolyzed in similar fashion to compound **6**, yielding (3*S*,5*R*)-(-)-3,5-dimethyldodecanoic acid (**10**) in 46% yield. $[\alpha]_D^{20}$= −19.2 º (c=0.48, CH_2Cl_2). ^{1}H NMR, ^{13}C NMR, IR, and mass spectra matched those of the corresponding (3*R*,5*S*)-enantiomer (**6**).

*Methyl (3S,5S)-(-)-3,5-Dimethyldodecanoate (**11**)* This diastereomer was made in analogous fashion in 84% isolated yield from methyl (2*E*,5*S*)-(-)-5-methyl-2-dodecenoate (**4**) (diastereomeric purity, 97.5% by GC), using (*S*)-Tol-BINAP as catalyst for the asymmetric addition reaction. $[\alpha]_D^{20}$=−2.2° (c=1.54, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, *J*=6.8 Hz, 3 H), 0.91 (d, J=9.2 Hz, 3 H), 0.93 (d, *J*=6.4 Hz, 3 H), 1.03 (m, 2 H), 1.27 (m, 12 H), 1.45 (br, 1 H), 2.05 (m, 2 H), 2.31 (m, 1 H), 3.67 (s, 3 H). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ 14.3, 20.3, 20.6, 22.9, 27.0, 28.1, 29.6, 30.1, 30.2, 32.1, 36.9, 41.8, 44.9, 51.5, 174.0. MS: *m/z* (%): 242 (2), 227 (1), 211 (2), 185 (11), 166 (3), 143 (9), 125 (3), 111 (7), 101 (73), 83 (17), 74 (100), 55 (32), 43 (54). IR (film) 2966, 2906, 2848, 2257, 1740, 1464, 1441, 1373, 1165, 1010, 913, 838, 733 cm^{-1}.

Laboratory Bioassays of Synthetic Pheromone We tested the responses of male *P. californicus* to synthetic pheromone using a rectangular olfactometer (80×10×19.5 cm, L × H × W) constructed of polyethylene with a removable glass top (see Cervantes et al., 2006). Both openings, at one end of the olfactometer were fitted with PVC female adapters (3.75 cm diam), to which we attached containers (17.8 cm-long sections of 3.75 cm diam clear PVC pipe, covered with aluminum screen at one end, and the other end inserted into a screened PVC male adapter) that held sample or control odor sources. Air was pulled (3.2 $m.s^{-1}$) from outside the

building through an activated charcoal filter, then through the sample containers and into the olfactometer chamber.

We conducted bioassays in July 2008 under a 25-W incandescent red light to minimize the influence of visual signals, during the 2nd to 6th hr of scotophase, when adult beetles typically are active (Barbour et al., 2007). All males walked rather than attempting to fly in the olfactometer. A positive score was given to males that walked upwind and reached a sample container that held the pheromone lure within 5 min. A negative score was given to those that reached the control container within 5 min. Beetles that did not reach either container within 5 min were scored "no response". We washed the olfactometer with soap (1–2% Micro-90®, Cole Parmer Instrument Co., Vernon Hills, IL, USA) and water after every five trials, then rinsed it with acetone, allowed it to air dry, and switched sample containers between sides to control for location effects. We tested at least five males against each treatment/dose and repeated the bioassays on at least 3 d ($N \geq 15$ for each treatment).

We tested the response of male *P. californicus* to synthetic 3,5-dimethyldodecanoic acid in the olfactometer using 1, 10, or 100 ng of material in 10 µl hexane (HPLC grade, Chromasolv®, Sigma-Aldrich, St. Louis, MO, USA). A filter paper disc (1.5 cm diam., Grade 413, VWR, West Chester, PA, USA) was placed on a piece of clean aluminum foil (~2.5 cm^2) and pheromone solution applied to the paper. The aluminum foil square and disc were placed into a randomly-selected sample container. A piece of aluminum foil and paper disc treated with 10 µl of hexane (control) was placed in the other container. Bioassays on each day were conducted using the lowest dose (1 ng) first, and then sequentially higher doses, so as to minimize any effects from contamination of the olfactometer by a previous treatment.

We compared the responses of males to (3*R*,5*S*)- and (3*S*,5*R*)-dimethyldodecanoic acid in the olfactometer using the methods described above. We tested 0.25, 2.5, and 25 ng of each enantiomer, corresponding to the approximate amount of each of the four stereoisomers in the 1, 10, and 100 ng range of doses of 3,5-dimethyldodecanoic acid that previously had been tested. To further evaluate the activity of (3*S*,5*R*)-dimethyldodecanoic acid, we ran bioassays using the lowest dose that had elicited a significant positive response from males to the (3*R*,5*S*)-enantiomer (2.5 ng; see Results) tested against a solvent control. The two diastereomers, (3*R*,5*R*)- and (3*S*,5*S*)-dimethyldodecanoic acid, were not tested in laboratory or field bioassays because their GC retention times were different from that of the insect-produced pheromone, conclusively eliminating them as candidates for the pheromone (see below). The mixture of four 3,5-dimethyldecanoic acid stereoisomers was synthesized as previously described (Rodstein et al., 2009), and the syntheses of the stereoisomers are described above.

Field Bioassays of Synthetic Pheromone Field bioassays of 3,5-dimethyldodecanoic acid and (3*R*,5*S*)-dimethyldodecanoic acid were conducted in southern California and Idaho. Our primary study site in southern California was the Santa Rosa Plateau Ecological Reserve (SRPER; Riverside Co.), a 5,600-ha area with extensive stands of mature oaks (*Quercus agrifolia* Née and *Q. engelmannii* Greene), coastal sage scrub, and chaparral. Adult *P. californicus* are commonly attracted to lights at the visitor center (SRPER staff, pers. comm.). Field bioassays at SRPER were conducted 2–6 July 2008 (maximum air temperatures 28–35°C, no precipitation). Field bioassays also were conducted in commercial hop yards (4–17 ha) in Canyon County, Idaho, that were naturally infested with *P. californicus* (unpub. data). Bioassays in Idaho were conducted 8–15 July 2008 (maximum air temperatures 28–36°C, no precipitation).

Traps used in California consisted of ~1.9-l wide-mouth plastic jars (P.E.T.; model 55–650, General Bottle Supply Company, Los Angeles, CA, USA), fitted with a 1.9-l plastic funnel, with the spout cut off to leave a ~3.5 cm diam hole at the bottom. A ~3.5 cm diam hole was cut into the threaded lid of the jar, and the funnel spout inserted into the hole and glued in place so that the spout was inside the jar. We treated interior surfaces of funnels and jars with Rain-X® glass treatment (Houston, TX, USA) to render them slippery. Traps were buried so that the rim of the funnel was nearly flush with the ground and, as required, a ramp to the funnel rim was built from soil and leaf litter. Lures were suspended ~15 cm over the trap with a stiff wire that was attached to the funnel. It was not possible to set up traps in linear transects at SRPER because of the abundant large rocks and boulders, so they were positioned arbitrarily under the canopy of oaks, at least ~10 m apart.

Traps in Idaho consisted of 19-l polypropylene buckets (38 cm tall × 30 cm diam) fitted with aluminum funnels (model 2815B, BioQuip, Rancho Dominguez, CA, USA). Traps were buried 90 m apart, within rows of hop plants, with the tops of funnels flush with the soil surface. Lures were suspended over funnels by attaching them to the wire bucket handles, which had been modified to remain upright.

Lures for traps in both California and Idaho consisted of clear low density polyethylene press-seal bags (Bagette model 14770, 5.1×7.6 cm, 0.05 mm wall thickness, Cousin Corp., Largo, FL, USA). Dilutions of synthetic pheromone or solvent were pipetted into the bags, which then were sealed. Experiments were set up in late afternoon or dusk, and treatments were assigned randomly to traps. Beetles were collected from traps the next morning or the morning

of the second day. In both locations, captured beetles were released into vegetation, at least 25 m away from the nearest trap, immediately after they were collected from traps.

Treatments in bioassays conducted at SRPER in southern California were as follows: 3,5-dimethyldodecanoic acid (10 mg in 100 μl hexane), (3*R*,5*S*)-dimethyldodecanoic acid (2.5 mg in 100 μl hexane), (3*S*,5*R*)-dimethyldodecanoic acid (2.5 mg in 100 μl hexane), and control (100 μl hexane). Treatments were assigned randomly to four traps in each of four blocks (at least 400 m apart) on 2 and 3 July 2008. We recorded the number of male *P. californicus* in traps (no females were captured), and rotated traps one position within blocks after the first day. Males of the congeneric species, *Prionus lecontei* Lameere, also were caught in traps at the southern California site, and trap captures recorded.

Lure doses in bioassays conducted in Idaho were as follows: 3,5-dimethyldodecanoic acid (1 mg in 100 μl hexane), (3*R*,5*S*)-dimethyldodecanoic acid (250 μg in 100 μl hexane), and control (100 μl hexane). We also included positive control traps, which were baited with a single live female *P. californicus*, to compare responses of males to natural *versus* synthetic pheromone. Unmated females were reared as described above, and were confined in a wire-mesh cage attached to the wire handle of trap buckets, analogous to the positioning of the synthetic lures. Treatments were randomized in each of four different hop yards on 14 July 2008. Captured males were collected from traps the next morning. The experiment was replicated on 15 July 2008 with treatments re-randomized, and using fresh females.

Dose–response trials with the synthetic pheromone were conducted in both southern California and Idaho. At SRPER in California, five traps in each of the four blocks (see above) were assigned randomly to lures containing 0, 10, 32, 100, and 320 μg of 3,5-dimethyldodecanoic acid in 100 μl of hexane. The experiment was set up on 4 July 2008 and run for three consecutive days (treatments rotated each day). In Idaho, we conducted two dose–response experiments, each in the same four hop yards. In the first (high-dose) experiment, traps were baited with 0, 10, 100, 1000, or 10^4 μg of the synthetic mixture of isomers (in 100 μl hexane) on 8 and 9 July 2008. The second (low-dose) experiment tested the responses of male beetles to 0, 0.1, 1, 10, and 100 μg in 100 μl hexane on 10 and 11 July 2008.

Statistics For laboratory bioassays, we compared the number of males that responded positively to treatments *versus* controls, or to one of the two enantiomers, with χ^2 goodness-of-fit tests. For field experiments, differences between treatments in numbers of beetles captured were tested separately for *P. californicus* and *P. lecontei* with the nonparametric Friedman's test (blocking by replicate; PROC FREQ with CMH option; SAS Institute, 2001), because assumptions of analysis of variance were violated by heteroscedasticity (Sokal and Rohlf, 1995). We tested differences between pairs of means with the REGWQ means-separation test to control maximum experiment-wise error rates (SAS Institute, 2001). Replicates that captured fewer than three beetles across all treatments were dropped from the analyses for each species.

Voucher specimens of the two *Prionus* species have been submitted to the W. F. Barr Entomology Museum at the University of Idaho and the Entomology Research Museum at the University of California, Riverside.

Results

Identification of the Insect-produced Stereoisomer 3,5-Dimethyldodecanoic acid, with two chiral centers, has four possible stereoisomers. The mixture of the four was separated cleanly into two GC peaks on an achiral DB-5 column, that corresponded to the *syn* (3*R**,5*R**) and *anti* (3*R**,5*S**) diastereomeric pairs of enantiomers (Rodstein et al., 2009). The corresponding methyl esters eluted in the same order as the parent acids and were also resolved to baseline, with the additional advantages that the peaks were more symmetrical and the retention times less variable than those of the parent acids. The retention times of the insect-produced acid and its methyl ester matched those of the later-eluting diastereomer of the synthesized acid and its methyl ester, respectively. These were proven to be the *anti*-diastereomers by comparing their retention times with those of *anti* (3*S*,5*R*)-3,5-dimethyldodecanoic acid and its ester; as expected, the retention times of *syn* (3*S*,5*S*)-dimethyldodecanoic acid and its ester matched those of the earlier eluting peaks in the synthetic mixtures of all four stereoisomers of the acid and their esters, respectively. However, we were not able to determine the absolute configuration of the insect-produced compound by analytical methods, because all attempts at resolving the methyl esters of the enantiomers on a chiral stationary phase GC column (Cyclodex-B) were unsuccessful. Thus, laboratory and field bioassays were used to indirectly determine the naturally-produced enantiomer.

Stereoselective Syntheses of Stereoisomers of 3,5-Dimethyldodecanoic Acid (Fig. 1) Three of the four possible stereoisomers of 3,5-dimethyldodecanoic acid were synthesized in good stereoisomeric purity, using the copper-catalyzed asymmetric conjugate addition of alkylmagnesium bromides to α,β-unsaturated methyl esters

(Lum et al., 2008) as the key reaction for introducing each of the two chiral methyl groups. Thus, (*E*)-2-decenoic acid (**1**) was converted to the corresponding methyl ester (**2**) by treatment with HCl in methanol, generated *in situ*. The first chiral methyl group then was introduced by reaction of α,β-unsaturated ester (**2**) with methylmagnesium bromide, with CuI and (*S*)-Tol-BINAP catalysis, to give methyl (*S*)-(-)-3-methyldecanoate (**3**). This compound was reduced to the corresponding aldehyde with DIBAL-H in hexane, followed by stabilized Wittig olefination in the same pot with methyl (triphenylphosphoranylidene) acetate to yield methyl (*S*)-(-)-5-methyl-2-dodecenoate as a mixture of (*Z*)- and (*E*)-isomers. These were separable by flash chromatography, giving the geometrically pure methyl (2*E*,5*S*)-(-)-5-methyl-2-dodecenoate (**4**) required for the next step. α,β-Unsaturated ester (**4**) then was subjected to the same copper-catalyzed asymmetric conjugate addition conditions with methylmagnesium bromide, CuI, and (*R*)-Tol-BINAP, yielding methyl (3*R*,5*S*)-(-)-3,5-dimethyldodecanoate (**5**). Hydrolysis of the ester with KOH in aqueous ethanol gave the free acid (**6**).

The enantiomeric (3*S*,5*R*)-(+)-3,5-dimethyldodecanoic acid (**10**) was made in analogous fashion by the same sequence of reactions, but using the catalysts with the opposite configuration to those used in the first sequence. Thus, unsaturated ester (**2**) was reacted with methylmagnesium bromide, CuI, and (*R*)-Tol-BINAP to give methyl (*R*)-(-)-3-methyldecanoate (**7**). DIBAL-H reduction and chain extension with methyl (triphenylphosphoranylidene) acetate yielded methyl (*R*)-(-)-5-methyl-2-dodecenoate (**8**). Asymmetric conjugate addition of compound **8** with methylmagnesium bromide, CuI, and (*S*)-Tol-BINAP yielded methyl (3*S*,5*R*)-(+)-3,5-dimethyldodecanoate (**9**), which was hydrolyzed to give (3*S*,5*R*)-(+)-3,5-dimethyldodecanoic acid (**10**).

Finally, reaction of methyl (2*E*,5*S*)-(-)-5-methyl-2-dodecenoate (**4**) with methylmagnesium bromide, CuI, and (*S*)-Tol-BINAP, gave the diastereomeric methyl (3*S*,5*S*)-(-)-dimethyldodecanoate (**11**).

Laboratory Bioassays of Synthetic Pheromone In olfactometer bioassays, 18 of 22 male *P. californicus* (82%) responded positively to 100 ng of 3,5-dimethyldodecanoic acid, and 36 of 41 males (88%) responded to 10 ng (treatments different from 1:1 positive:negative responses for both: $\chi^2_{1,\ 22}=21.8$, $P=0.003$; $\chi^2_{1,\ 41}=23.4$, $P<0.001$, respectively). However, only 13 of 22 (62%) males responded to 1 ng of 3,5-dimethyldodecanoic acid (treatment not different from 1:1: $\chi^2_{1,\ 21}=1.19$, $P=0.28$).

When equal doses of the two enantiomers were directly compared in olfactometer bioassays, 13 of 14 males (93%) and 13 of 16 males (81%) moved to the olfactometer arm baited with (3*R*,5*S*)- dimethyldodecanoic acid, at doses of 25 and 2.5 ng, respectively (treatments different from 1:1; $\chi^2_{1,\ 14}=10.3$, $P=0.001$; $\chi^2_{1,\ 16}=6.25$, $P=0.012$). Males showed a marginal positive response to (3*R*,5*S*)- over 3*S*,5*R*-dimethyldodecanoic acid at 0.25 ng (8 of 10 males [80%]; $\chi^2_{1,\ 10}=3.6$, $P=0.058$). Males did not respond to either 0.25 or 2.5 ng of (3*S*,5*R*)-dimethyldodecanoic acid when this enantiomer was tested against a solvent control (6 of 13 males [48%] and 10 of 17 [59%]: not different from 1:1; $\chi^2_{1,\ 25}=0.04$, $P=0.84$; $\chi^2_{1,\ 17}=0.53$, $P=0.47$, respectively).

Field Bioassays of Synthetic Pheromones Traps in southern California baited with 3,5-dimethyldodecanoic acid, (3*R*,5*S*)-dimethyldodecanoic acid, or (3*S*,5*R*)-dimethyldodecanoic acid, captured a total of 100 male *P. californicus* and 23 male *P. lecontei*. No females of either species were caught. The numbers of male *P. californicus* and *P. lecontei* captured were strongly and similarly affected by experimental treatment (Friedman's $Q_{3,\ 28}=18.2$, $P<0.001$, $Q_{3,16}=9.4$, $P=0.025$, respectively). Male *P. californicus* responded strongly and equally to 3,5-dimethyldodecanoic acid and (3*R*,5*S*)-dimethyldodecanoic, but showed little attraction to (3*S*,5*R*)-dimethyldodecanoic acid or controls (Fig. 2). Male *P. lecontei* were most strongly attracted to (3*R*,5*S*)-dimethyldodecanoic acid, showed intermediate attraction to 3,5-dimethyldodecanoic acid, and were not attracted to (3*S*,5*R*)-dimethyldodecanoic acid or controls (Fig. 2).

Traps baited with 3,5-dimethyldodecanoic acid, (3*R*,5*S*)-dimethyldodecanoic acid, or live female *P. californicus* in Idaho captured a total of 252 male *P. californicus*, but no *P. lecontei*. Trap catches of male *P. californicus* again were affected by experimental treatment (Fig. 3; Friedman's $Q_{3,31}=23.4$, $P<0.001$). 3,5-Dimethyldodecanoic acid and (3*R*,5*S*)-dimethyldodecanoic acid attracted similar numbers of males. Significantly fewer males were captured in traps baited with virgin females or in controls (Fig. 3). The latter

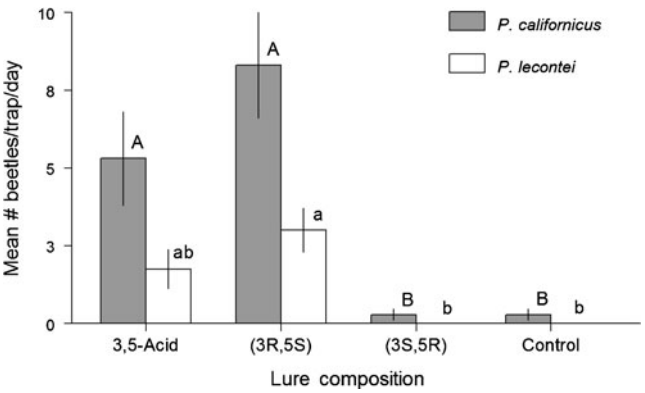


Fig. 2 Mean number (± SE) of male *Prionus californicus* (*N*=100) and *Prionus lecontei* (*N*=23) captured in southern California by traps baited with 3,5-dimethyldodecanoic acid ("3,5-Acid"), (3*R*,5*S*)-dimethyldodecanoic acid, (3*S*,5*R*)-dimethyldodecanoic acid, and solvent lures ("Control"). Bars with different letters of the same case are significantly different (REGWQ test, $P<0.05$)

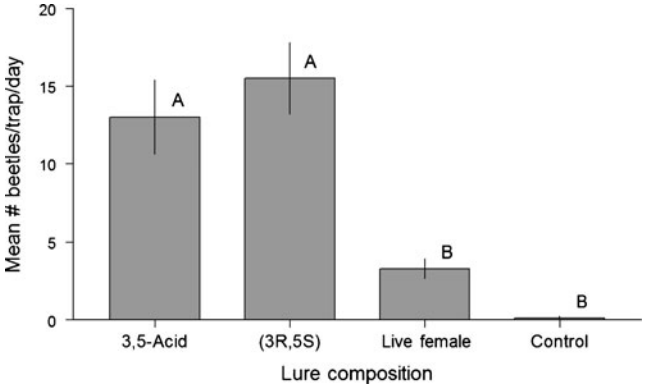


Fig. 3 Mean number (± SE) of *Prionus californicus* males (N=252) captured in Idaho by traps baited with racemic 3,5-dimethyldodecanoic acid ("3,5-Acid", 1 mg), (3*R*,5*S*)-dimethyldodecanoic acid (0.25 mg), a live female *Prionus californicus*, and control lures treated with solvent ("Control"). Bars with different letters are significantly different (REGWQ test, P<0.05)

two treatments did not differ in the number of beetles captured (Fig. 3).

In the dose–response bioassay in southern California, traps captured totals of 62 male *P. californicus* and 44 male *P. lecontei.* There were too few replicates that captured three or more *P. lecontei* to allow a robust statistical analysis. For *P. californicus*, trap catch was affected by dose (Fig. 4, top; Friedman's $Q_{4,50}$=17.2, P=0.002), with only traps in the 320 μg treatment capturing more beetles than control traps. The high- and low-dose trials for the dose–response bioassays in Idaho captured 145 and 139 male *P. californicus*, respectively, and the number of males captured was affected by pheromone dose in both trials (Fig. 4, bottom; Friedman's $Q_{4,30}$=21.2, P<0.001, $Q_{4,45}$= 32.0, P<0.001, respectively). The two trials were consistent in capturing no beetles in control traps, and in traps baited with doses higher than 100 μg (high-dose trial) or 10 μg (low-dose trials) capturing more male *P. californicus* than control traps (Fig. 4, bottom). The number of males captured did not differ for pheromone doses of 0.1 mg and higher in the high-dose trial, but in the low-dose trial, the number of males captured was higher for 100 μg than for 1 and 10 μg (Fig. 4, bottom).

Discussion

Identification and Synthesis of the Stereoisomers To verify the identification of the basic carbon skeleton of the pheromone, we previously had developed a nonstereoselective synthesis of the mixture of all four stereoisomers of 3,5-dimethyldodecanoic acid (Rodstein et al., 2009). Although the diastereomers or their methyl esters were readily separable on GC, we did not know which diastereomer corresponded to which peak. Thus, we stereoselectively synthesized three of the four possible stereoisomers, with the goal of first determining the relative stereochemistry of the methyl groups in the pheromone (i.e., the correct diastereomer), followed by determination of the natural enantiomer. With these standards in hand, we showed unequivocally that the insect-produced compound was the *anti* diastereomer. Although we were not able to resolve the enantiomers (as their methyl esters) on a chiral stationary phase GC column, the question as to which enantiomer was produced by the insects was resolved indirectly by the results of both laboratory and field bioassays, in which (3*R*,5*S*)-3.5-dimethyldodecanoic acid was highly attractive to male beetles, whereas its enantiomer was virtually inactive.

The enantiomers of 3,5-dimethyldodecanoic acid were synthesized using the asymmetric conjugate addition developed by Lum et al. (2008) to place each of the chiral methyl groups. Use of this method has several

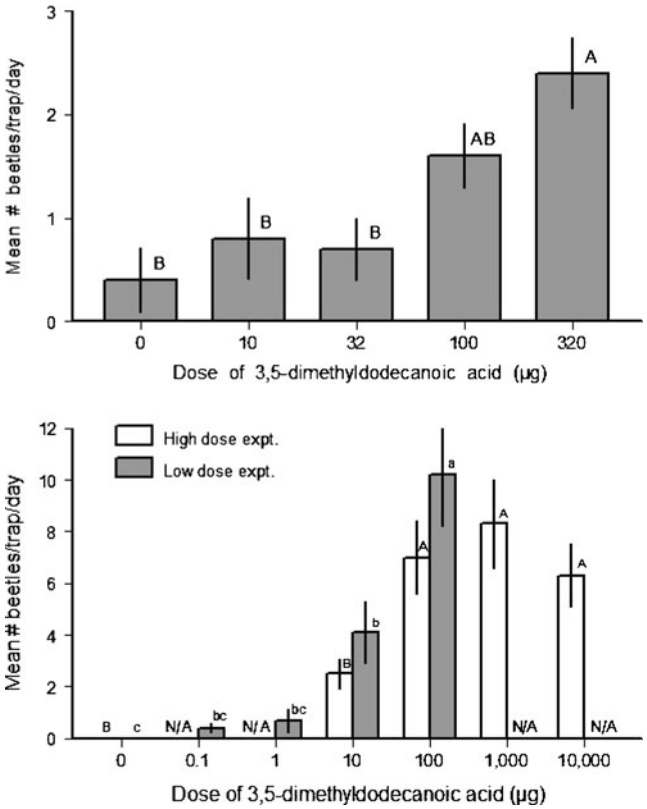


Fig. 4 Mean number (± SE) of adult male *Prionus californicus* captured in traps baited with various doses of racemic 3,5-dimethyldodecanoic acid. *Top*; bioassays conducted in California (N= 62); *Bottom*: bioassays conducted in Idaho testing high and low doses (N=145 and 139 males, respectively). N/A indicates dosages that were not included in a particular experiment. Bars with different letters of the same case are significantly different (REGWQ test, P<0.05)

advantages. First, each conjugate addition step proceeds with high enantioselectivity, yielding products of known absolute configuration. Second, either enantiomer is equally accessible simply by changing the chiral ligand in the reaction (i.e., (*R*)-Tol-BINAP or (*S*)-Tol-BINAP). Third, the reaction can be applied readily in iterative fashion, so that the same reaction can be used to place each methyl group sequentially. However, this route was not without minor problems. For example, it proved to be critically important to use the α, β-unsaturated methyl esters for the addition reaction; use of the analogous ethyl esters resulted in a decrease in stereoselectivity.

During the course of these reactions, all direct attempts at determining the enantiomeric purities of intermediates, final products, or several derivatives thereof, were unsuccessful. Thus, enantiomeric pairs of methyl esters **3** and **7**, **4** and **8**, and **5** and **9** were not resolved on the Cyclodex-B column, nor were the corresponding alcohols from reduction of these esters resolved. Furthermore, the enantiomers of 1-bromo-2-methylnonane, derived from hydrolysis of methyl 3-methyldecanoate followed by decarboxylative halogenation by the Cristol-Firth modification of the Hunsdieker reaction (Cristol and Firth, 1961), failed to resolve on this column, and the analogous enantiomers of 1-bromo-2,4-dimethylundecane also were not resolved. Whereas the diastereomeric purities of the (3*S*,5*R*)-, (3*R*,5*S*)-, and (3*S*,5*S*)-stereoisomers were 92.5, 90.7, and 97.5%, respectively, as determined by the ratios of their GC peak areas, the enantiomeric purities must necessarily be much higher because almost all of the enantiomeric impurity formed in the first asymmetric reaction is converted to a diastereomer of the desired product in the second asymmetric reaction. Similarly, any loss of stereospecificity in the second asymmetric reaction results in a diastereomer of the desired product rather than the undesired enantiomer.

Bioassay Results The fact that only males of *P. californicus* were attracted to synthetic pheromone in field bioassays confirmed that (3*R*,5*S*)-dimethyldodecanoic acid is a female-produced sex pheromone for this species. This compound does not function as an aggregation pheromone, as appears to be the case for the male-produced pheromone of many species in the subfamilies Aseminae and Cerambycinae (see Introduction). Male *P. californicus* were quite sensitive to the pheromone, as indicated by the 0.25–2.5 ng response threshold in the olfactometer bioassays. This level of sensitivity is consistent with the strong responses of males to live females, dead females, and even their excised ovipositors, demonstrated in an earlier study (Cervantes et al., 2006). The response was entirely enantiospecific: males showed no response in laboratory or field studies to the unnatural enantiomer, (3*S*,5*R*)-dimethyldodecanoic acid. There was no indication that any of the unnatural stereoisomers inhibited behavioral responses to the insect-produced stereoisomer, with male *P. californicus* responding as well to the mixture of the four stereoisomers as they did to (3*R*,5*S*)-dimethyldodecanoic acid. This strong attraction to the mixture of stereoisomers will be useful for commercial development of the pheromone because it circumvents the need for an expensive chiral synthesis.

Dose–response bioassays in the field demonstrated that males responded to doses of synthetic pheromone as low as 10 μg when dispensed from plastic bag dispensers, consistent with their responses to minute quantities of pheromones in olfactometer bioassays. As doses increased, the numbers of males captured reached a plateau midway through the series, with doses >100 μg attracting similar numbers of males. Traps baited with the synthetic pheromone captured greater numbers of males than did the caged females used as positive controls. In previous studies, we demonstrated that traps baited with live females capture large numbers of males when not competing with pheromone traps (Cervantes et al., 2006). Taken together, these results suggest that the pheromone of this species consists of (3*R*,5*S*)-dimethyldodecanoic acid as a single component, despite the fact that extracts from females contain both homologs (with longer chains) and analogs (methyl esters of the acids) of 3,5-dimethyldodecanoic acid (Rodstein et al., 2009).

Our capture of male *P. lecontei* in southern California in traps baited with the pheromone of *P. californicus* was unexpected, and suggests that females of *P. lecontei* also produce a sex pheromone that contains (3*R*,5*S*)-dimethyldodecanoic acid. To date, we have not been able to confirm this, because all attempts at collecting female *P. lecontei* at light or light traps have been fruitless. The dose–response profile of male *P. lecontei* to (3*R*,5*S*)-dimethyldodecanoic acid was similar to that of male *P. californicus*, which further suggests that this compound is a major, if not the only, component of the pheromones of both species. What remains unclear is how these congeners maintain reproductive isolation, because they are sympatric in at least some areas of southern California (Monné and Hovore, 2005), and clearly have overlapping annual flight periods (i.e., substantial numbers of males of both species were caught during field trials in midsummer). It is possible that one or more of the other components in the crude extracts from *P. californicus* females may serve to inhibit the responses of male *P. lecontei*. This possibility remains to be tested once the other components have been synthesized and are available for follow-up field trials.

The complete absence of *P. lecontei* in Idaho may have been due to our sampling only in hop yards, or because southwest Idaho lies outside the geographic range of *P. lecontei*. Both *P. lecontei* and *P. californicus* are distributed in western North America, but there is little information available about their altitudinal distribution, beyond the fact that *P. californicus* occurs in the Rocky Mountains, whereas *P. lecontei* appears to be limited to a more western distribution (Linsley, 1962). Overall, these results indicate that traps baited with 3,5-dimethyldodecanoic acid will be an ideal tool to characterize the distribution and abundance of these congeners over their entire ranges.

The strong attraction of male *P. californicus* to even small amounts of synthetic pheromone also suggests that this compound has potential for monitoring and managing this species in agricultural and horticultural situations, where it is an important pest. As described above, *P. californicus* is one of the most important pests of hop in the Pacific Northwest. In addition, *P. californicus* and *P. lecontei* are chronic pests of a variety of tree crops, including apples and stone fruits (Solomon, 1995). We currently are evaluating potential uses of the pheromone for control of *P. californicus* in hop production by mating disruption or mass trapping.

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Purification, Stereoisomeric Analysis and Quantification of Sex Pheromone Precursors in Female Whole Body Extracts from Pine Sawfly Species

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Abstract A GC-MS method to analyze the stereoisomeric composition of chiral secondary alcohols found in whole body extracts of pine sawfly females was developed. The tested alcohols were derivatized with optically pure (*S*)-2-acetoxypropionyl chloride prior to GC-MS analysis. Baseline separation was obtained for all sixteen stereoisomers of 3,7,9-trimethyltridecan-2-ol and for the four 3-methylpentadecan-2-ol stereoisomers. For 3,7-dimethyltridecan-2-ol, 3,7-dimethyltetradecan-2-ol and 3,7-dimethylpentadecan-2-ol baseline separation was obtained for 6 of the possible 8 stereoisomers. When a mixture of 16 stereoisomers of 3,7,11-trimethyltridecan-2-ol was tested, baseline separation of 7 peaks out of 16 possible was obtained. The investigated alcohols are pheromone precursors for some pine sawfly species that are severe defoliators of pine. Females from several *Diprion, Neodiprion, Macrodiprion, Microdiprion*, and *Gilpinia* species emit esters of such secondary alcohols as sex pheromones that attract males for mating. To quantify the small amounts of the precursor alcohol and its stereoisomeric composition found in whole body extracts from female pine sawflies, a purification method was optimized. An extract of 20 females of *D. pini* contained about 8 ng of (2*S*,3*R*,7*R*)-3,7-dimethyltridecan-2-ol per female, and three extracts of 18, 20, and 90 females of *N. sertifer* contained between 5 and 13 ng of (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol per female.

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Key Words Sex pheromone · Pine sawflies · *Diprion pini* · *Neodiprion sertifer* · *Gilpinia frutetorum* · *Gilpinia pallida* · *Gilpinia socia* · *Macrodiprion nemoralis* · *Microdiprion pallipes* · 3,7-dimethyltridecan-2-ol · 3,7,9-trimethyltridecan-2-ol · 3,7,11-trimethyltridecan-2-ol · 3,7-dimethyltetradecan-2-ol · 3,7-dimethylpentadecan-2-ol · 3-methylpentadecan-2-ol · Stereoisomers · Analysis · GC-MS

Introduction

Pine sawflies of the family Diprionidae (Hymenoptera: Symphyta) are severe defoliators of pines in the Northern hemisphere (Smith, 1993). Larvae consume the needles, which results in reduced growth or even death of the trees. Most of these species belong to the genera *Diprion* or *Neodiprion*, but also members of other genera such as *Gilpinia*, *Macrodiprion*, and *Microdiprion* cause damage (Bergström et al., 1995; Anderbrant et al., 2005). Forest outbreaks of pine sawflies normally are controlled by chemical insecticides. With increased environmental concern, however, there is a growing interest in finding less harmful methods. Females of several pine sawfly species emit sex pheromones that attract males for mating. An alternative method to control and reduce the population is to use the insects own sex pheromone for monitoring, mass capture or mating disruption (Östrand et al., 1999; Rieske et al., 2001; Martini et al., 2002). The pheromone is stored as an alcohol precursor in the female body, which is esterified at the moment of release from the insect (Jewett et al., 1976; Anderbrant et al., 2005). Females from *Neodiprion sertifer* have the acetate or propionate of (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol as the main component of their sex pheromone (Anderbrant et al., 2000). In this case, esters of (2*S*,3*R*,7*R*)-3,7-dimethylpentadecan-2-ol act as antago-

nists, and decrease the male response from esters of (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol, even at concentrations as low as 0.1% (Anderbrant et al., 1992). Total inhibition of the response is obtained at concentrations above 2% of esters of (2*S*,3*R*,7*R*)-3,7-dimethylpentadecan-2-ol (Anderbrant et al., 1992). Thus, it is crucial to have a sensitive analytical method to quantify and determine the composition of the stereoisomers in females. Diastereomers, such as (2*S*,3*R*,7*R*)- and (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol, have very small differences in their physical properties, and separating them directly by using standard GC chiral columns is difficult. Enantiomers, such as (2*R*,3*R*,7*R*)- and (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol, can either be separated directly on a chiral column, or indirectly by using a chiral derivatizing reagent (Gübitz and Schmid, 2001; Srinivas, 2004) to form diastereomers. Stereoisomeric analysis of secondary alcohols has been accomplished via derivatization with chiral acid chlorides, benzoyl chloride, or isopropyl isocyanate, sometimes in combination with chiral columns (Brooks et al., 1973; Doolittle and Heath, 1984; Högberg et al., 1990; Wassgren and Bergström, 1995). Pre-purification of whole body extracts from pine sawflies prior to derivatization normally is necessary, and often is done with Solid Phase Extraction on a SiOH-column with gradient elution (Bergström et al., 1995; Wassgren and Bergström, 1995; Östrand et al., 2003).

The aim of the present study was to develop a method, based on chiral derivatization and analysis by GC-MS, by which stereoisomers of chiral secondary alcohols found in whole body extracts from female pine sawflies can be determined and quantified. The method was developed by using pure stereoisomers or mixtures of synthetic 3,7-dimethyltridecan-2-ol, 3,7,9-trimethyltridecan-2-ol, 3,7,11-trimethyltridecan-2-ol, 3,7-dimethyltetradecan-2-ol, 3,7-dimethylpentadecan-2-ol, and 3-methylpentadecan-2-ol as references. The alcohols selected for the study are used as pheromone precursors in *Diprion pini*, *Macrodiprion nemoralis*, *Microdiprion pallipes Gilpinia pallida*, *N. sertifer*, *Gilpinia frutetorum*, and *Gilpinia socia*. For demonstration, the method was applied on whole body extracts from *D. pini* and *N. sertifer*. Figure 1 shows the structures of all the alcohols thus far identified in pine sawfly species (Bergström et al., 1995; Tai et al., 1998; Anderbrant et al., 2000; Wassgren et al., 2000; Östrand et al., 2003; Hedenström et al., 2006, 2009).

Methods and Materials

Chemicals Solvents used for purification and derivatization were of spectrophotometric grade or higher, and purchased from Sigma-Aldrich, Schnelldorf, Germany. (*S*)-2-Acetoxypropionyl chloride (*Fluka Chemicals* Ltd.) and 2-naphthoyl chloride (98%) also were purchased from Sigma-Aldrich. The 100 mg Teflon coated Orochem Silica Superclean solid phase columns were purchased from Dalco Chromtech AB, Sollentuna, Sweden, and the 500 mg Strata SI-1 Silica Teflon coated solid phase columns from Skandinaviska Genetec AB, Västra Frölunda, Sweden. All alcohols, in pure stereoisomeric forms or in mixtures, previously had been synthesized in our laboratory (Högberg et al., 1990; Bergström et al., 1995; Hedenström et al., 2002). Thus, some of the reference alcohols were used as pure stereoisomers and some as different mixtures of isomers to develop the separation method.

Extract Whole bodies of insect females were extracted in ethyl acetate according to Wassgren et al. (1992). To an extract, corresponding to 21 female *D. pini* (France), 200 ng of 2-tridecanol were added as an internal standard for quantification. From the same original *D. pini* sample, to an amount corresponding to 20 females, 198 ng 2-tridecanol and 355 ng of (2*R**,3*S**,7*R*/*S*)-3,7-dimethyltridecan-2-ol [1:1:1:1 mixture of the isomers (2*R*,3*S*,7*R*), (2*R*,3*S*,7*S*), (2*S*,3*R*,7*S*), and (2*S*,3*R*,7*R*)] were added. To three different extracts of *N. sertifer* (Uppland and Värmland, Sweden), between 200 and 873 ng (depending on the number of insects in the extracts) of 2-pentadecanol were added as an internal standard.

Purification Gradient elution on solid phase columns was used for purification of extracts (Bergström et al., 1995; Wassgren and Bergström, 1995; Östrand et al., 2003). The method was optimized with a reference mixture of 3,7-dimethyltridecan-2-ol and 2-tridecanol (used as internal standard). To eliminate co-eluting contaminants from ordinary polypropylene columns, Teflon coated columns were used. For extracts of 1–10 insects, a 100 mg Teflon coated Orochem Silica Superclean solid phase column was used, and a 500 mg Strata SI-1 Silica Teflon coated solid phase column was used for extracts of more than 10 insects. The column was cleaned and conditioned with three column volumes each of ethyl acetate, 10% ethyl acetate in pentane, and pentane. For large or contaminated samples, the extract was divided into smaller portions (100–200 ng), purified separately, and then combined. Internal standard was added to the extract before purification. 2-Tridecanol was used as internal standard for the analysis of 3,7-dimethyltridecan-2-ol (*D. pini*), and 2-pentadecanol for the analysis of 3,7-dimethylpentadecan-2-ol (*N. sertifer*). The extract solution was evaporated to dryness under a stream of argon at room temperature, and resolved in 100–200 μl of pentane. The solution was purified on the solid phase column with gradient elution, stepwise with 1–11% ethyl acetate in pentane in steps of 1% (fractions 1–11). An elution and collection volume of 150 μl for each fraction

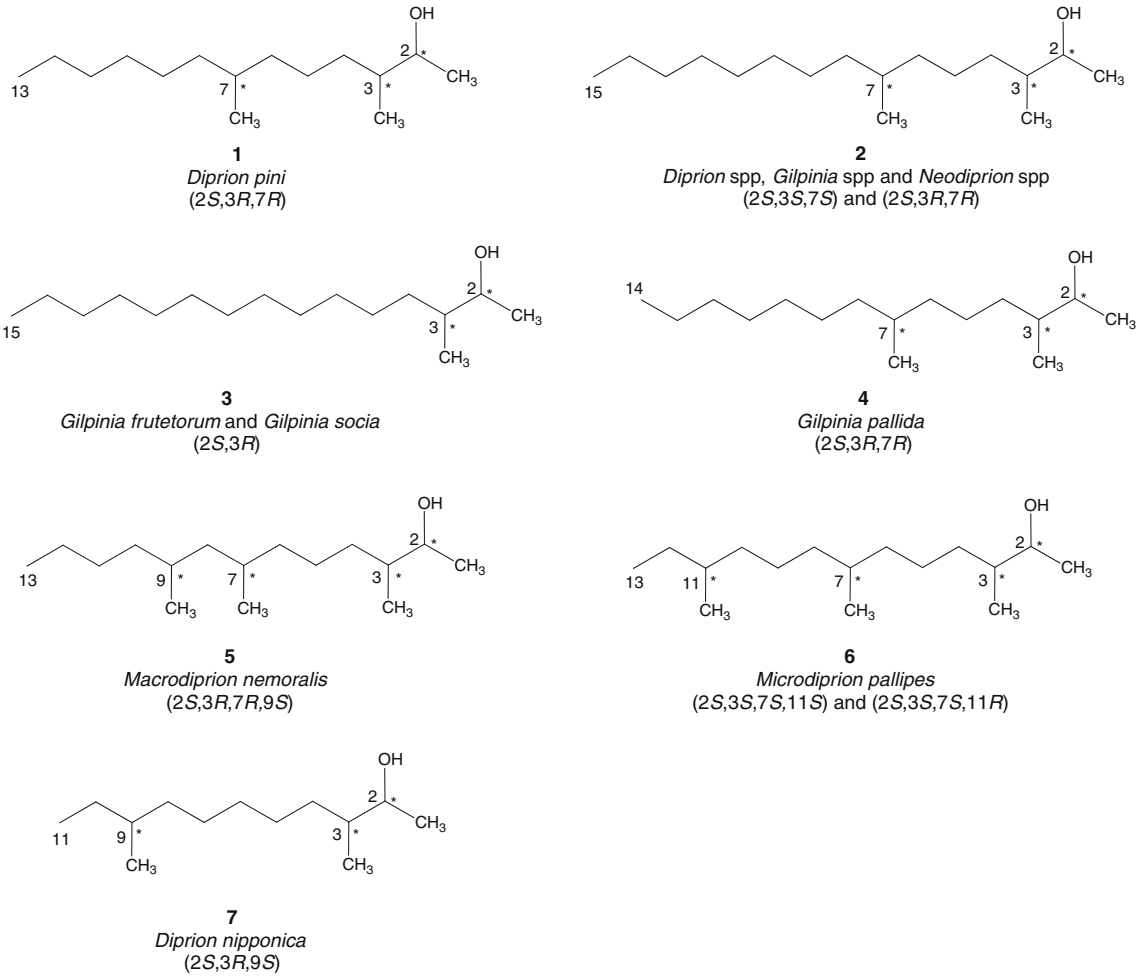


Fig. 1 Structures of the chiral alcohols that have been identified in pine sawfly species as sex pheromone precursors

was used for 100 mg columns with twice the elution and collection volume for fractions 9–11, and 750 µl for 500 mg columns with twice the elution and collection volume for fractions 9–11. The later fractions, containing the alcohols as judged by GC, were collected and combined in a vial and evaporated to dryness under a stream of argon at room temperature. Pentane (1 ml) was added, and the solution was washed with 200 µl of 0.2 M KOH. The organic layer was collected with a Pasteur pipette, and dried by passing through a second Pasteur pipette containing dry sodium sulphate, a piece of glass wool that was rinsed with ethyl acetate, and pentane, and thereafter dried before use. The obtained alcohol containing solution was evaporated to dryness under a stream of argon at room temperature, and resolved in 50 µl cyclohexane. An injection of the sample was made on GC-MS for identification of the alcohols, and thereafter the remaining sample was derivatized as below.

Derivatization The reference solutions and the purified extracts in 50 µl cyclohexane were derivatized with (*S*)-2-acetoxypropionyl chloride (Doolittle and Heath, 1984) by adding 65 µl of 1% pyridine in cyclohexane (containing 3 Å molecular sieves), and then 100 µl of 1% (*S*)-2-acetoxypropionyl chloride in dichloromethane were added under a stream of argon. The solution was mixed and heated for 30 min in an oil bath at 80°C with a needle through the vial septum and then evaporated to dryness with argon through an additional needle. Pentane (1 ml) and 0.2 ml 1 M HCl (aq) were added to the vial, and the solution mixed. The organic layer was collected with a Pasteur pipette, dried by passing through a new Pasteur pipette containing dry sodium sulphate as above. The solution was evaporated to dryness under a stream of argon at room temperature, and the residue redissolved in 50 µl cyclohexane.

Derivatization of (2*S*,3*S*,7*R*)- and (2*S*,3*S*,7*S*)-3,7-dimethyltridecan-2-ol with 2-naphthoyl chloride was made according to the method used in Wassgren and Bergström (1995) for other acid chlorides, but with some modifications. The mixture was heated for 60 min on an oil bath at 70°C, 10 µl methanol were added, and the solution was kept in the oil bath for an additional 5 min to transform the excess of naphthoyl chloride to its methyl ester. The

solution was evaporated to dryness with argon in the oil bath. H_2O (20 μl) was added, and the formed ester extracted with 2×100 μl pentane. The organic phase was washed with 2×10 μl H_20, and dried with sodium sulphate as previously.

Gas Chromatography and Mass Spectrometry The GC-MS analysis was performed on a Hewlett-Packard 6890N (GC), and an HP 5973 mass spectrometer (MS) with electron impact (EI) ionization. The carrier gas, 1 ml/min, was helium; 1 μl of the sample was injected splitless, the injector temperature was 250°C, and the aux temperature was 280°C.

Different GC-columns were evaluated with a stereoisomeric mixture of (2*R**,3*S**,7*R/S*)-3,7-dimethyltridecan-2-ol for optimal separation efficiency: ALPHA-DEX 120, BETA-DEX 120 and GAMMA-DEX 120 (all chiral) from Supelco, HP-1ms (non-polar) and HP-5ms (non-polar) from Agilent Technologies, factorFOUR VF-23ms (polar) from Varian (all 30 m×0.25 mm i.d., d_f=0.25 μm), CP-Sil 88 (polar) from Chrompack (50 m×0.25 mm i.d., d_f= 0.20 μm), and HP-88 (polar) from Agilent Technologies (100 m×0.25 mm i.d., d_f=0.20 μm). The factorFOUR VF-23ms column was chosen for method development due to good separation in combination with high inertness and low bleed.

For identification of the alcohols in purified extracts, the column temperature was increased from 50°C by 10°C/min up to 230°C. Full scan mode was used, and the retention times and MS-spectra of the eluting peaks were compared with reference samples. For separation and identification of the stereoisomers of the reference samples and extracts derivatized with (*S*)-2-acetoxypropionyl chloride, selected ion monitoring (SIM) was used to maximize sensitivity and reduce the number of interfering peaks. Temperature gradients and the ions for SIM mode were selected according to Table 1. The ions *m/z* 87, 115, and 133 originate from the (*S*)-2-acetoxypropionyl part (m/z 115), with *m/z* 87=115–CO and m/z 133 [due to double hydrogen rearrangement i.e., McLafferty + 1 (McLafferty, 1959)]. The other selected ions originate from the alcohol part: *m/z* 210 from 3,7-dimethyltridecan-2-ol, *m/z* 224 from 3-methylpentadecan-2-ol, 3,7-dimethyltetradecan-2-ol, 3,7,9-trimethyltridecan-2-ol, and 3,7,11-trimethyltridecan-2-ol, and *m/z* 238 from 3,7-dimethylpentadecan-2-ol (See Table 1 for mass spectra data). For the analysis of extracts with internal standards, *m/z* 182 from 2-tridecanol (*D. pini*) and *m/z* 210 from 2-pentadecanol (*N. sertifer*) also were selected.

For the references derivatized with 2-naphthoyl chloride, the temperature gradient and the ions for SIM mode were selected according to Table 1. The ions *m/z* 127, 155, and 172 originate from the 2-naphthoyl part (*m/z* 155), with *m/z* 127=155–CO and *m/z* 172 [due to McLafferty rearrangement (McLafferty, 1959)]. The *m/z* 210 ion originate from the alcohol part and *m/z* 382 the molecular ion (See Table 1 for mass spectra data).

Results

Purification Experiments The purification method was optimized by eluting a reference mixture of 3,7-dimethyltridecan-2-ol and 2-tridecanol (internal standard) on the SiOH column, with 1–15% ethyl acetate in pentane in steps of 1%, and by collecting and analyzing the fractions by GC-MS. The best result for collecting the alcohol was achieved when eluting with 9–11% of ethyl acetate in

Table 1 Temperature gradients and SIM ions for the analysis of secondary alcohol samples

Alcohol	Temperature gradient	Selected ions (*m/z*) and (%) for SIM
3-methylpentadecan-2-ol[a]	50–125°C: 10°C/min, 125–131°C: 0.01°C/min, 131–230°C: 10°C/min	87 (36), 115 (100), 133 (14), 224 (4)
3,7-dimethyltridecan-2-ol[a]	50–120°C: 10°C/min, 120–126°C: 0.01°C/min, 126–230°C: 10°C/min	87 (47), 115 (100), 133 (23), 210 (10)
3,7-dimethyltetradecan-2-ol[a]	50–120°C: 10°C/min, 120–126°C: 0.01°C/min, 126–230°C: 10°C/min	87 (43), 115 (100), 133 (28), 224 (9)
3,7-dimethylpentadecan-2-ol[a]	50–125°C: 10°C/min, 125–131°C: 0.01°C/min, 131–230°C: 10°C/min	87 (42), 115 (100), 133 (25), 238 (7)
3,7,9-trimethyltridecan-2-ol[a]	50–120°C: 10°C/min, 120–126°C: 0.01°C/min, 126–230°C: 10°C/min	87 (47), 115 (100), 133 (36), 224 (5)
3,7,11-trimethyltridecan-2-ol[a]	50–120°C: 10°C/min, 120–126°C: 0.01°C/min, 126–230°C: 10°C/min	87 (46), 115 (100), 133 (30), 224 (13)
(2S,3S,7R)- and (2S,3S,7S)-3,7-dimethyltridecan-2-ol[b]	50–145°C: 10°C/min, 145–151°C: 0.01°C/min, 151–230°C: 10°C/min	127 (32), 155 (100), 172 (96), 210 (3), 382 (4)

[a]Derivatized with (*S*)-2-acetoxypropionyl chloride and analysed on the HP-88 column

[b]Derivatized with 2-naphthoyl chloride and analysed on the factor FOUR VF-23ms column

pentane, using twice the dead volume of the column for these fractions which contained the alcohol. This was repeated with 3,7-dimethylpentadecan-2-ol and 2-pentadecanol (internal standard), which gave the same good result.

Derivatization Experiments A reference solution of (2*R**, 3*S**,7*R/S*)-3,7-dimethyltridecan-2-ol diluted in cyclohexane was derivatized with (*S*)-2-acetoxypropionyl chloride. The method was optimized by varying the molar ratio of 3,7-dimethyltridecan-2-ol and (*S*)-2-acetoxypropionyl chloride between 1:3 and 1:100, the reaction time between 10 and 60 min, and the temperature of the oil bath between 40°C and 80°C. A molar ratio of 1:10 and a reaction time of 30 min at 80°C were found to be sufficient. The optimized method was used for derivatization of all different alcohols.

Separation Experiments Out of the eight different GC-columns that were tested (ALPHA-DEX 120, BETA-DEX 120, GAMMA-DEX 120, HP-1ms, HP-5ms, factorFOUR VF-23ms, CP-Sil 88, and HP-88) by using a standard solution of (2*R**,3*S**,7*R/S*)-3,7-dimethyltridecan-2-ol derivatized with (*S*)-2-acetoxypropionyl chloride, the HP-88

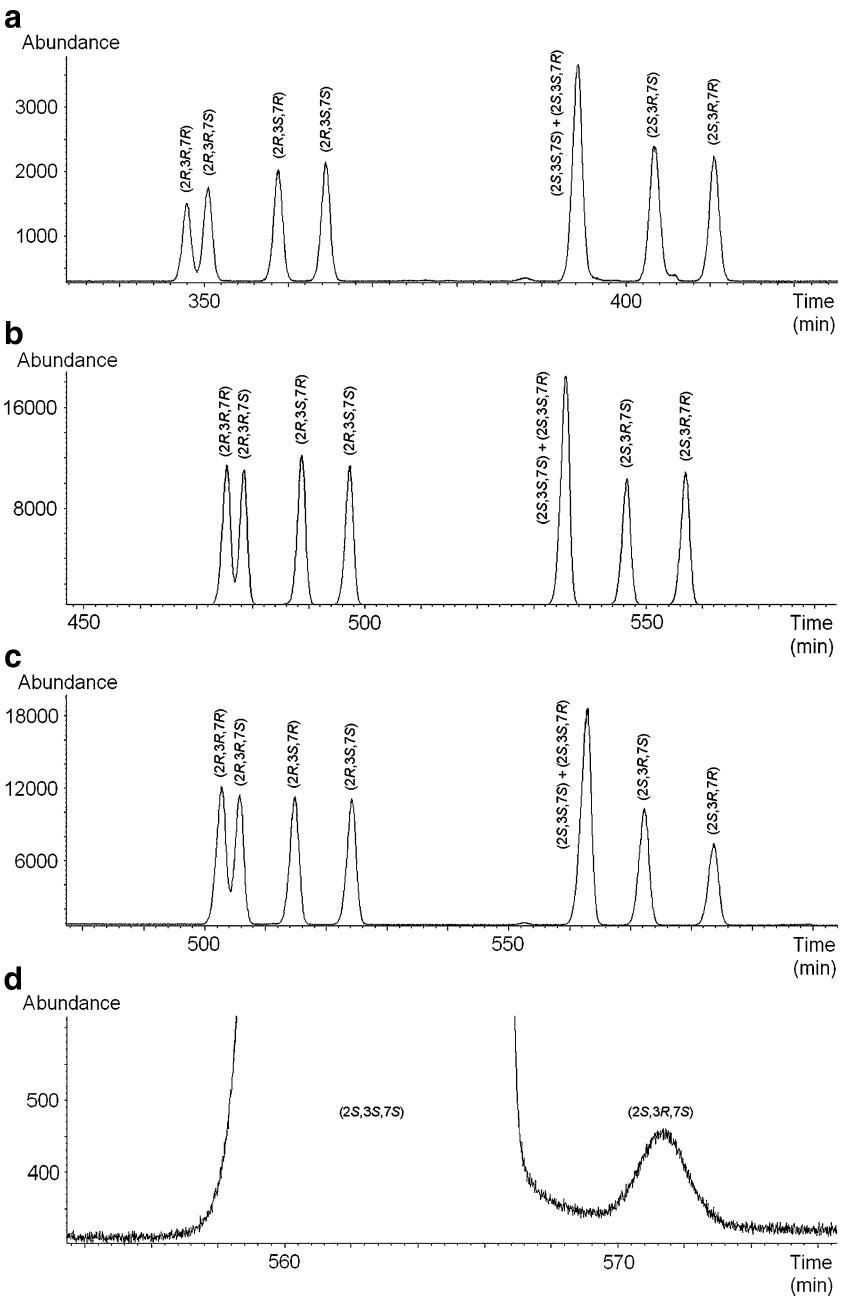
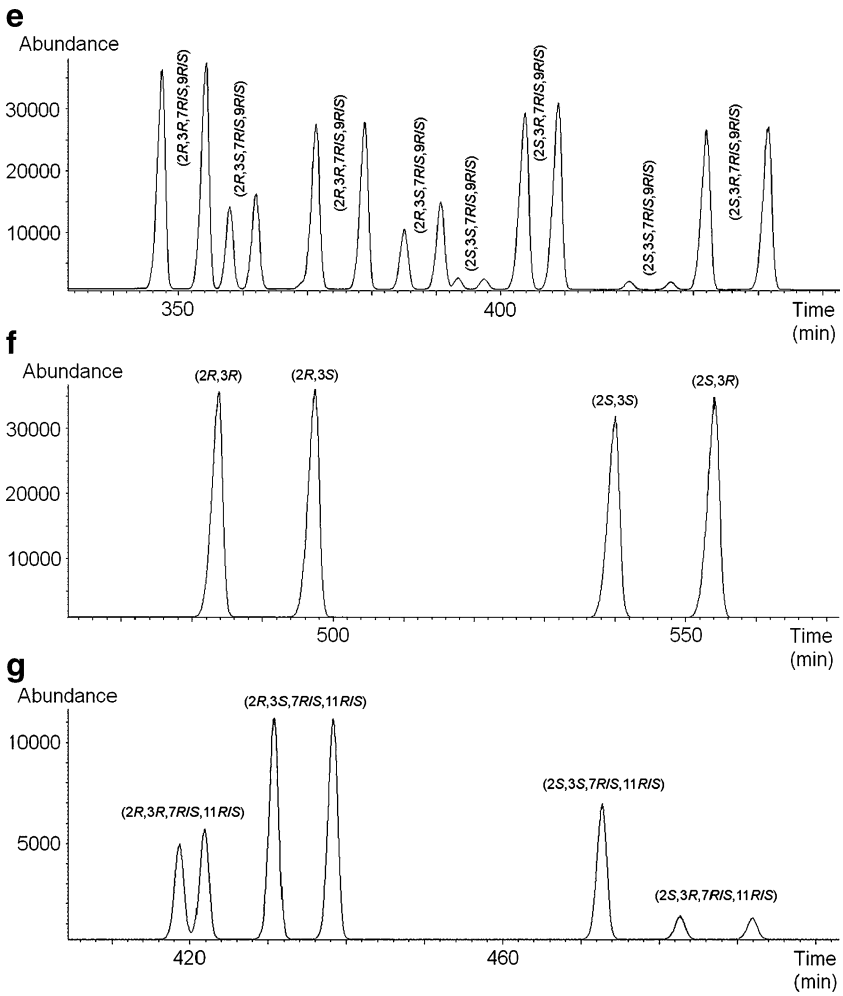


Fig. 2 GC-MS chromatogram after derivatization with (*S*)-2-acetoxypropionyl chloride of the stereoisomers of; **a** 3,7-dimethyltridecan-2-ol; **b** 3,7-dimethyltetradecan-2-ol; **c** 3,7-dimethylpentadecan-2-ol; **d** (*2S,3S,7S*)- containing 0.1% (*2S,3R,7S*)-3,7-dimethylpentadecan-2-ol; **e** 3,7,9-trimethyltridecan-2-ol (the individual peaks were not assigned to a corresponding stereoisomer); **f** 3-methylpentadecan-2-ol; **g** 3,7,11-trimethyltridecan-2-ol (the individual peaks were not assigned to a corresponding stereoisomer). See Table 1 for details

Fig. 2 (continued)



gave the best separation. Since it is not bonded or cross-linked and relatively expensive, however, it was used only for the optimized methods for the analysis of synthetic reference mixtures of the precursor alcohols and the extracts. For the development of the purification and derivatization methods, the factor FOUR VF-23ms column was used, because of its higher inertness and longer life time, and a separation performance that was close to that for the HP-88 column.

Of the eight stereoisomers of 3,7-dimethyltridecan-2-ol, 3,7-dimethyltetradecan-2-ol and 3,7-dimethylpentadecan-2-ol, respectively, the four (2*R**,3*S**,7*R*/*S*)-isomers, separated easily into four peaks with baseline separation (Fig. 2a–c). For the four (2*R**,3*R**,7*R*/*S*)-isomers, [(2*R*,3*R*,7*R*), (2*R*,3*R*,7*S*), (2*S*,3*S*,7*S*), and (2*S*,3*S*,7*R*)] the (2*R*,3*R*,7*R*)- and (2*R*,3*R*,7*S*)-isomers separated, on the HP-88 column almost with baseline separation, but no separation at all was achieved for the (2*S*,3*S*,7*S*)- and (2*S*,3*S*,7*R*)-isomers (Fig. 2a–c).

An experiment also was made by derivatizing a reference compound (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol containing 0.1% of the (2*S*,3*R*,7*S*)-isomer. The injected amount of (2*S*,3*R*,7*S*) was 83 pg, and its peak was separated clearly from the peak of (2*S*,3*S*,7*S*) (Fig. 2d).

Baseline separation of all the 16 isomers was obtained when a, derivatized, reference mixture of 16 stereoisomers of 3,7,9-trimethyltridecan-2-ol with (*S*)-2-acetoxypropionyl chloride derivatized was analysed (Fig. 2e). Similarly, all four stereoisomers of 3-methylpentadecan-2-ol gave good baseline separation (Fig. 2f).

For the 16 stereoisomeric mixture of 3,7,11-trimethyltridecan-2-ol, the result was baseline separation of seven peaks, but separation of all individual isomers was not possible with this method (Fig. 2g).

Because complete separation of (2*S*,3*S*,7*R*)- and (2*S*,3*S*,7*S*)-3,7-dimethyltridecan-2-ol isomers derivatized with (*S*)-2-acetoxypropionyl chloride was not accomplished, we used 2-naphthoyl chloride as a derivatizing reagent instead. This gave baseline separation between the peaks on the factor FOUR VF-23ms column (Fig. 3). However, with this method some of the other isomers co-eluted (not shown).

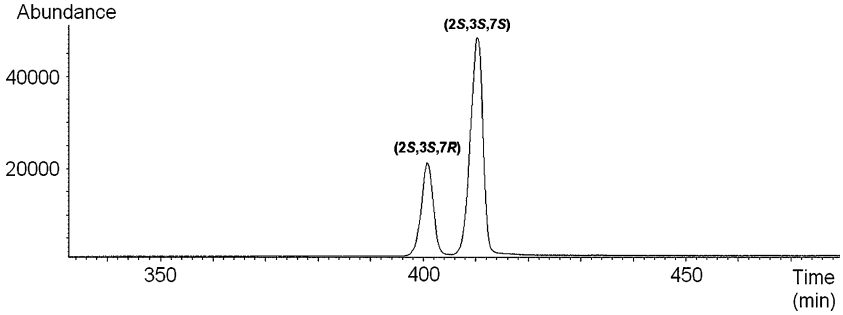


Fig. 3 GC-MS chromatogram of (2*S*,3*S*,7*R*)- and (2*S*,3*S*,7*S*)-3,7-dimethyltridecan-2-ol after derivatization with 2-naphthoyl chloride. See Table 1 for details

Analysis of Extracts The extract of female *D. pini* (France) contained about 8 ng of (2*S*,3*R*,7*R*)-3,7-dimethyltridecan-2-ol per female (Fig. 4, Table 2). The amount was calculated by adding a known amount of *(2R*,3S*,7R/S)*-3,7-dimethyltridecan-2-ol to one part of the sample and comparing the area of the (2*S*,3*R*,7*R*)-peak with the areas of the three other compounds.

The three extracts of *N. sertifer* (Sweden) contained between 5 and 13 ng of (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol per female (Fig. 5 and Table 2). The amounts were calculated by comparing the area of the (2*S*,3*S*,7*S*)-peaks with the area of the internal standard and correcting for difference in response and molar weight. The correction factor was calculated by running known amounts of the internal standard and synthetic references of (2*S*,3*S*,7*S*)- and (2*S*,3*R*,7*S*)-3,7-dimethylpentadecan-2-ol through the whole method, and comparing the areas in the GC-MS-chromatograms.

No other stereoisomers of 3,7-dimethyltridecan-2-ol or 3,7-dimethylpentadecan-2-ol were detected in the extracts of *D. pini* and *N. sertifer*. The small peaks seen in Figs. 4 and 5 did not exhibit the correct SIM ion ratio for the insect pheromone components.

Discussion

When analyzing the (*S*)-2-acetoxypropionyl esters of 3,7-dimethyltridecan-2-ol, 3,7-dimethyltetradecan-2-ol, and 3,7-dimethylpentadecan-2-ol, six of the eight stereoisomers were separated. However, (2*S*,3*S*,7*R*) and (2*S*,3*S*,7*S*) stereoisomers were not separated. A previous method, with the 3,7-dimethylpentadecan-2-ol as isopropyl carbamate derivatives tested on a chiral XE-60-(*S*)-valine-(*S*)-2-phenylethylamide column, also separated the *(2R*,3S*,7R/S)*-isomers as four peaks and the *(2R*,3R*,7R/S)*-isomers as two peaks (Högberg et al., 1990). However, this method also gave overlapping peaks between the *(2R*,3S*,7R/S)*- and *(2R*,3R*,7R/S)*-isomers. The researchers tried to improve the separation by coupling three different non-chiral columns in series and by converting the alcohol to derivatives of benzoate (Högberg et al., 1990) or pentafluorobenzoate (Wassgren and Bergström, 1995), but despite these efforts the stereoisomers (2*S*,3*S*,7*R*) and (2*S*,3*S*,7*S*) were only partly separated. By using 2-naphthoyl chloride we obtained baseline separation between the peaks of the diastereomeric esters (2*S*,3*S*,7*R*)- and (2*S*,3*S*,7*S*)-3,7-dimethyltridecan-2-ol. (Fig. 3). This also can be applied to 3,7-dimethyltetradecan-2-ol, 3,7-dimethylpentadecan-2-ol, and perhaps, even 3,7,9-trimethyltridecan-2-ol, since they all have similar separation patterns. As this method gives co-elution of other peaks instead, it can be seen as a complement to derivatizing with (*S*)-2-acetoxypropionyl chloride.

As mentioned in the introduction, even very low concentrations of esters of the (2*S*,3*R*,7*R*)-isomer in the female sex pheromone can affect the response behavior of the receiving male to the emitted esters of (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol. Thus, we chose to test (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol, with 0.1% of the (2*S*,3*R*,7*S*)-isomer added, as this represents an even more difficult separation compared to the (2*S*,3*S*,7*S*)-isomer with a small amount of (2*S*,3*R*,7*R*)-isomer, since a large peak easily overlaps a closely later eluting and smaller peak

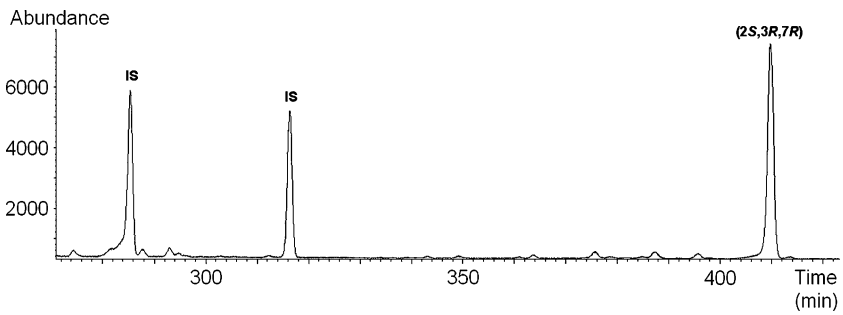


Fig. 4 GC-MS chromatogram of an extract of 21 female *Diprion pini* (France) after derivatization with (*S*)-2-acetoxypropionyl chloride. See Tables 1 and 2 for details

Table 2 Extracts of female pine sawfly species

Specie	Location	Number of insects	Amount of IS added (ng)	Amount of SRR in extract (ng/female)	Amount of SSS in extract (ng/female)
Diprion pini	France (1994)	21/20[a]	200/198[a,b]	8	–
Neodiprion sertifer	Värmland, Sweden (1991)	18	200[c]	–	12
Neodiprion sertifer	Uppland, Sweden (1991)	20	200[c]	–	13
Neodiprion sertifer	Uppland, Sweden (1993)	90	873[c]	–	5

IS internal standard, *SRR* (2*S*,3*R*,7*R*)-3,7-dimethyltridecan-2-ol, *SSS* (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol

[a]The same sample divided into two parts, one (n=20, IS=198 ng) spiked with a reference mixture of 355 ng *(2R*,3S*,7R/S)*- 3,7-dimethyltridecan-2-ol

[b]2-tridecanol

[c]2-pentadecanol

(Fig. 2c). Even for this demanding task our method gave excellent separation using the HP-88 column, and the result is shown in Fig. 2d.

An extraordinary result was obtained with our method when the reference mixture of 16 stereoisomers of 3,7,9-trimethyltridecan-2-ol showed, after derivatization with (*S*)-2-acetoxypropionyl chloride, good separation of all the 16 isomers (Fig. 2e). Although, we here just show the elution order and have not assigned each individual peak in the spectra to the corresponding stereoisomer, the results demonstrate that it is possible to efficiently analyze the stereoisomeric composition of the sex pheromone precursor content of females from *M. nemoralis*.

For the derivatized 16 stereoisomeric mixture of 3,7,11-trimethyltridecan-2-ol, the result was only partial separation, with no separation of individual isomers (Fig. 2g). Although the only difference between 3,7,9-trimethyltridecan-2-ol and 3,7,11-trimethyltridecan-2-ol is the positioning of one of the methyl groups, the chromatographic separation is different. It is known that other compounds with one methylene group between two methyl groups, i.e., in our case 3,7,9-trimethyltridecan-2-ol, show unique biological and physical properties when compared to compounds without this structure (i.e., in our case 3,7,11-trimethyltridecan-2-ol) (Hoffmann, 1992).

The quantitative analysis of female extracts from *D. pini* and *N. sertifer* presented herein showed results similar to previous studies, with 8 ng per female of (2*S*,3*R*,7*R*)-3,7-dimethyltridecan-2-ol for *D. pini* (Bergström et al., 1995) and 5–13 ng per female of (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-ol for *N. sertifer* (Wassgren et al., 1992; Wassgren and Bergström, 1995). No other stereoisomers were detected in the extracts. By using MS detection with SIM in our method, the chromatograms showed few co-eluting contaminants in the area of interest and, thereby, the results should be easy to repeat accurately.

The described method is an efficient way to analyze all of the isomers of the secondary pheromone precursor alcohols, 3,7,9-trimethyltridecan-2-ol and 3-methylpentadecan-2-ol, in extracts of female pine sawflies. For 3,7-dimethyltridecan-2-ol, 3,7-dimethyltetradecan-2-ol, and 3,7-dimethylpentadecan-2-ol, baseline separation was obtained for six of the possible eight stereoisomers. To separate the (2*S*,3*S*,7*S*)- and (2*S*,3*S*,7*R*)-isomers, we showed that it is possible by using 2-naphthoyl chloride as the derivatization agent. Another way to obtain good separation between the (2*S*,3*S*,7*S*)- and (2*S*,3*S*,7*R*)-isomers is to use the (*R*)-isomer instead of the (*S*)-isomer of 2-acetoxypropionyl chloride, as the separation then will look like the result obtained with (2*R**,3*R**,7*R*/*S*)- and (2*R**,3*S**,7*R*/*S*)-isomers in Fig. 2a–c. Since (*R*)-2-

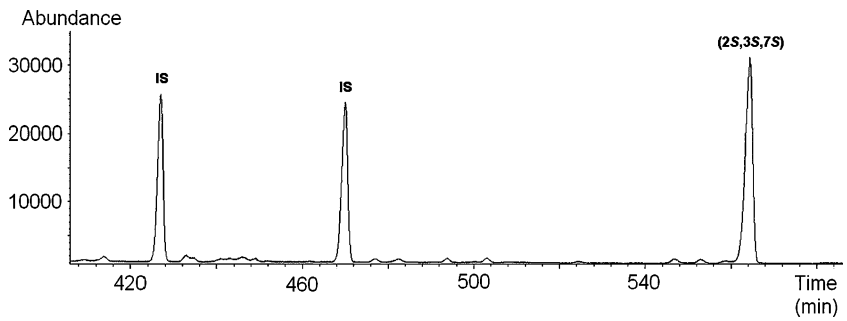


Fig. 5 GC-MS chromatogram of an extract from 90 *Neodiprion sertifer* females (Uppland, Sweden) after derivatization with (*S*)-2-acetoxypropionyl chloride. See Tables 1 and 2 for details

acetoxypropionyl chloride is not commercially available, however, it has to be synthesized in enatiomerically pure form. Until now, although, the (2*S*,3*S*,7*R*)-isomers of 3,7-dimethyltridecan-2-ol, 3,7-dimethyltetradecan-2-ol, and 3,7-dimethylpentadecan-2-ol have not been found in extracts of a female pine sawfly species.

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Sex Pheromone Components and Control of the Citrus Pock Caterpillar, *Prays endocarpa*, Found in the Mekong Delta of Vietnam

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Abstract The citrus pock caterpillar, *Prays endocarpa* (Yponomeutidae; Praydinae), is a pest of pomelo (*Citrus grandis* L.) in Vietnam. Gas chromatography-mass spectrometry analyses of pheromone gland extracts from female moths identified three monoenyl compounds, (*Z*)-7-tetradecenal (Z7-14:Ald), (*Z*)-7-tetradecenyl acetate (tentatively identified, Z7-14:OAc), and (*Z*)-7-tetradecen-1-ol (Z7-14:OH), in a ratio of about 10:3:10. In the field, traps baited with synthetic Z7-14:Ald (0.5 mg) caught male *P. endocarpa*. The other two compounds, either alone or when added to Z7-14:Ald, did not elicit increases in trap catch (relative to the appropriate treatment). Synthetic Z7-14:Ald was used to monitor and control this species in pomelo orchards in Vinh Long Province. Monitoring revealed that adults were present throughout the year with discernible peaks in December, March, and April. A mass-trapping trial, using 20 traps in a 0.1 ha pomelo orchard, effectively suppressed fruit damage to levels similar to that achieved by an insecticide (Karate 2.5EC). Mating disruption trials, using polyethylene-tube dispensers, each filled with 80 mg of Z7-14:Ald at a rate of 200 or 400 dispensers/ha, also controlled damage by this pest to levels below that achieved by an insecticide treatment. This work demonstrates the potential for pheromone-based control of this pest in Vietnam.

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Key Words Lepidoptera · Yponomeutoidea · (*Z*)-7-Tetradecenal · Synthetic pheromone · Male attractant · Monitoring · Mass trapping · Mating disruption

Introduction

The citrus pock caterpillar (CPC), *Prays endocarpa* Meyrick (Lepidoptera: Yponomeutidae: Praydinae), is one of the most serious pests of citrus orchards in South and Southeast Asia. While larvae of the congeners, *Prays citri* Milliere and *Prays nephelomima* Meyrick, feed on citrus flowers (the two species are known as citrus flower moths), larvae of CPC attack fruit exclusively, particularly those of pomelo, *Citrus grandis* L. After hatching, the larvae mine into the peel of fruit and feed. Pomelo fruit, attacked by CPC larvae at an early stage of development, will drop from the tree before ripening; fruit attacked at a later stage will develop tumors on the peel, which greatly decrease the commercial value of the fruit. The control of CPC by insecticides is difficult because sprayed insecticide does not directly contact the larvae in the fruit peel. In the Mekong Delta of Vietnam, pomelo trees have two main fruiting seasons: the first from February to May, and the second from September to November; pomelo fruit also is sporadically produced outside of these periods. Since pomelo fruit is expensive, often used as a special gift, control of this pest with no, or minimal, pesticide residue is highly desirable. We decided to identify the sex pheromone of this species in order to develop a control program based on synthetic sex pheromone.

Among species in the genus *Prays*, sex pheromones have been identified from the citrus flower moths, *P. citri* (Nesbitt et al., 1977) and *P. nephelomima* (Gibb et al., 2005), and the olive moth, *P. olea* Bern. (Campion et al.,

1979; Renou et al., 1979). These three species all utilize (*Z*)-7-tetradecenal (Z7-14:Ald) in their pheromonal communication. In this paper, we report the results of studies on chemical identification of compounds produced in the sex pheromone gland of female *P. endocarpa*, field trials testing the ability of these compounds to attract males, as well as the efficacies of mass trapping and mating disruption, using a polyethylene formulation of the synthetic pheromone, to prevent fruit damage by CPC.

Methods and Materials

Insect and Pheromone Extract Pomelo fruit infested with CPC larvae were collected in orchards in My Hoa and Dong Thanh villages, in Vinh Long Province of Vietnam, and brought to Can Tho University. In the laboratory, the fruit was placed in transparent plastic boxes and kept under light and temperature conditions similar to those outside (27°C–30°C, 55%–70% RH, 12L:12D) until larvae pupated. Pupae were collected and placed individually in a small glass tube. Abdominal tips of 2- or 3-d-old adult virgin females were excised 2 hr after the onset of the scotophase and extracted in HPLC-grade hexane (10 μl/female) for 15 min. Abdominal tip extracts [>250 female equivalent (FE)] were sent to Tokyo University of Agriculture and Technology for analysis by gas chromatography-mass spectrometry (GC-MS).

GC-MS Analysis Electron ionization (70 eV) mass spectra of the pheromone components and authentic standards were obtained on a Hewlett-Packard 5973 mass selective detector (Agilent Technologies Inc., Palo Alto, CA, USA), using a DB-23 column (0.25 mm i.d.×30 m, 0.25 μm film, J & W Scientific, Folsom, CA, USA), with helium as carrier gas. The column was programmed from 80°C (held for 1 min) to 220°C at 8°C per min and held for 10 min. To determine the position of a double bond in the compounds, dimethyl disulfide (DMDS) adducts were formed. Briefly, the crude pheromone extract (80 FE) was dissolved in a mixture of DMDS (50 μl) and a diethyl ether solution of iodine (60 mg/ml, 5 μl) and held at 40°C overnight (Buser et al., 1983; Vang et al., 2005). After addition of 10% sodium thiosulfate solution (0.5 ml), crude products were extracted with hexane and analyzed by GC-MS. Adducts were analyzed on the same instrument, using an HP-5 column (0.25 mm i.d.×30 m, 0.25 μm film, Agilent) and the same conditions and temperature program.

Synthesis of (Z)-7-Tetradecenal and Derivatives (*Z*)-7-Tetradecen-1-ol (Z7-14:OH) was synthesized from 1,7-heptanediol. Successive reactions with 3,4-dihydropyrane and pyridinium chlorochromate (PCC) converted the diol to the THP ether of 7-hydroxyheptanal, which was coupled with a phosphorane prepared from 1-bromoheptane and triphenylphosphine. This Wittig reaction produced the THP ether of Z7-14:OH accompanied by a small amount of the (*E*)-isomer. After cleavage of the THP ether, Z7-14:OH (>99% purity) was obtained by column chromatography using silica gel impregnated with 15% $AgNO_3$. Z7-14:Ald and (*Z*)-7-tetradecenyl acetate (Z7-14:OAc) were synthesized by oxidation with PCC and acetylation with acetic anhydride of the alcohol, respectively. For mating disruption, Z7-14:Ald (about 200 g) was prepared from Z7-14:OAc (Shin-Etsu Chemical Company, Tokyo, Japan) by hydrolysis with KOH in methanol, followed by Swern oxidation to the aldehyde.

Field Trapping of Male Moths A synthetic compound(s), dissolved in hexane, was applied to a rubber septum (8 mm o.d., white rubber; Aldrich Chemicals, Milwaukee, WI, USA). After evaporation of the solvent, the lure was placed at the center of a sticky trap (30×27 cm bottom plate, Takeda Chemical Ind., Ltd., Osaka, Japan), which was hung on a pomelo tree approximately 1.5 m from the ground. Captured males were counted weekly. For comparison of trap catches, virgin females (three, 2- or 3-d-old, allowed to feed on 10% sugar water absorbed on a cotton wick) were caged in a small tea sieve and placed in a trap; females were renewed every 2 d.

Field trials for monitoring and mass trapping of *P. endocarpa* males were carried out using the same traps baited with a lure containing 1.0 mg of Z7-14:Ald on a rubber septum. The monitoring experiment was carried out in pomelo orchards of three different villages in Vinh Long Province in 2007, with three traps placed in each pomelo orchard. Traps were checked roughly every second week. The mass trapping experiment was carried out in three 0.1 ha plots in each of My Hoa and Dong Thanh villages, in Vinh Long Province, from April 4 to June 10, 2008. The three plots in each village had different treatments: 20 pheromone-baited traps only, a pyrethroid insecticide (cyhalothrin, Karate 2.5EC) sprayed three times during the test, or no (control) treatment. In the pheromone treatment, trapped males were counted weekly, and each lure was renewed monthly over the course of the experiment. Fruit damaged by CPC larvae in plots of the orchards was determined by inspecting the peel, and was assessed three times, on May 15, May 25, and June 10.

Formulation of Polyethylene Dispensers and Evaluation of Mating Disruption Synthetic Z7-14:Ald and stabilizers (Sumisorb 300 and Sumilizer BHT, 3% each, Sumitomo Chemical Co., Ltd., Osaka, Japan) were poured into 20-cm-long red polyethylene tubes, which were used for "Hamaki-con®" (Shin Etsu Chemical Co). Each tube contained about 80 mg of the

aldehyde and was suspended by twisting it around a small branch of a pomelo tree, approximately 1.5–2.0 m high above the ground. The efficacy of mating disruption was evaluated over two consecutive pomelo fruiting seasons in Vinh Long Province. The first trial was carried out from August 15 to September 30, 2008, in plots of three separate orchards located >500 m from each other: a 0.75-ha plot using 300 tubes (400 tubes/ha), a 0.6-ha plot using 120 tubes (200 tubes/ha), and a 0.5-ha plot using no tubes (control). Insecticides were not sprayed in the orchards during the trial. The second trial was carried out from August 11 to October 30, 2009 in plots of four separate orchards: a 1.1-ha plot using 440 tubes (400 tubes/ha), a 0.8-ha plot using 160 tubes (200 tubes/ha), and 0.3-ha and 0.6-ha plots using no tubes (control). In the last plot, a pyrethroid insecticide (cyhalothrin, Karate 2.5EC®) was sprayed three times during the trial; no insecticides were sprayed in the other orchards. The trial areas used were mostly square, with pomelo trees planted at a density of 400–500 trees/ha.

The efficacy of mating disruption was assessed by counting trapped males and damaged fruit in the orchards. Three traps, baited with a lure (Z7-14:Ald, 1.0 mg/septum), were hung in the middle of each orchard, with the lures renewed monthly. Captured males were counted biweekly in 2008 and weekly in 2009. Fruit damaged by CPC larvae was assessed as for the mass trapping experiment. In each orchard, 24 trees in peripheral plots and 18 trees in central plots were marked with a red stripe. CPC infestation was examined for all the fruit developed during the test term on the marked trees.

Data Analysis Data obtained from the experiments were analyzed by one-way ANOVA and Tukey-Kramer's HSD test. Means were standardized using log(x+0.5) and arcsine {SQRT(x)} transformation, in order to homogenize the variance.

Results

GC-MS Analysis of Female Extract Extracts of CPC were analyzed by GC-MS (Fig. 1). While the extract included many unknown compounds (Fig. 1a), the mass chromatogram of *m/z* 192 ($[M\text{-}H_2O]^+$, Fig. 1c) suggested the occurrence of a tetradecenal at 11.45 min. The retention time (Rt) and mass spectrum of this compound was the same as that for authentic Z7-14:Ald. In addition to the aldehyde, a mass chromatogram of *m/z* 194 (Fig. 1b), suggested two (Rts of 12.68 and 12.89 min) other candidates for pheromone components. The Rts and mass spectra of these compounds were coincident with those of authentic Z7-14:OAc and Z7-14:OH, respectively. GC-MS analysis of the extract treated with DMDS confirmed the positions of the double bonds for Z7-14:Ald and Z7-14:OH. The DMDS adduct derived from the aldehyde component (Rt 22.45 min) showed M^+ at *m/z* 304 and fragment ions at *m/z* 145 and 159, while the DMDS adduct derived from the alcohol component (Rt 23.99 min), showed M^+ at *m/z* 306 and fragment ions at *m/z* 145 and 161. As the concentration of the acetate component was low, the mass spectrum of the DMDS adduct could not be identified. The *cis*-geometry of these compounds was confirmed by the respective retention times of the *E*-isomers on the DB-23 column: E7-14:Ald (11.30 min), E7-14:OAc (12.54 min), and E7-14:OH (12.73 min). A

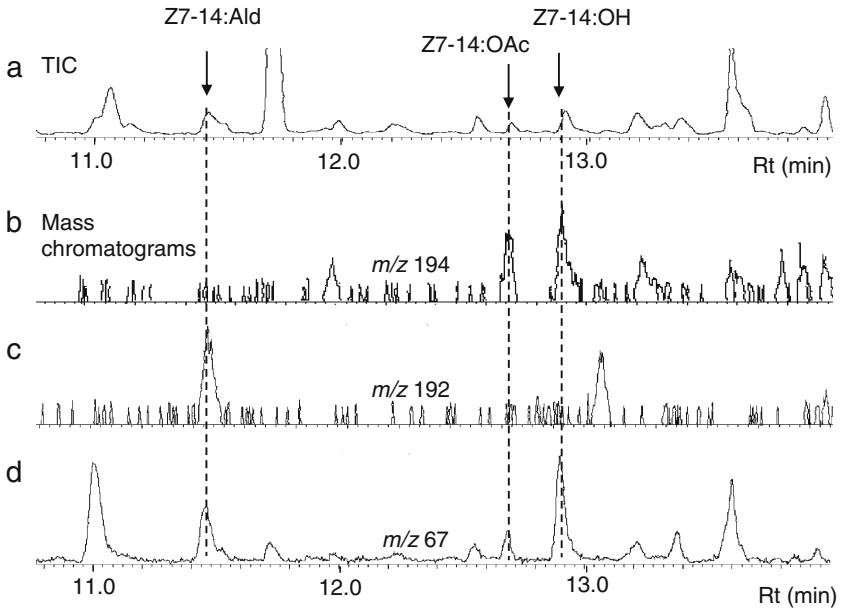


Fig. 1 Gas chromatography-mass spectrometry analysis of a pheromone gland extract from 30 female moths of the citrus pock caterpillar, *Prays endocarpa*. Total ion chromatogram (**a**), mass chromatograms at *m/z* 194 (**b**), 192 (**c**), and 67 (**d**)

mass chromatogram of *m/z* 67, a common fragment ion of tetradecenyl compounds, revealed no traces of *E*-isomers of any of the compounds (Fig. 1d). Mass spectral analysis of several extracts showed the ratio of Z7-14:Ald: Z7-14:OAc: Z7-14:OH to be about 10:3:10.

Optimization of the Synthetic Lure The three compounds, Z7-14:Ald, Z7-14:OH, and Z7-14:OAc were tested in field trials in pomelo orchards in My Hoa village from 2006 to 2009 (Table 1). Traps baited with Z7-14:Ald alone caught CPC males, while traps baited with other single-component lures, 1:1 binary blends, or a ternary blend did not (Table 1a). Addition of small amounts of Z7-14:OAc to Z7-14:Ald did not affect the catch of male CPC, relative to that caught by Z7-14:Ald alone. However, addition of a small amount of Z7-14:OH to Z7-14:Ald resulted in a drop in catch (relative to Z7-14:Ald alone), suggesting that Z7-14:OH inhibits the activity of Z7-14:Ald to CPC males (Table 1b). Increasing ratios of Z7-14:OAc to Z7-14:Ald in the blend resulted in decreased trap catches, although whether this was (at least in part) due to decreases of Z7-14:Ald amount was not investigated (Table 1c).

In general, increasing dosages of Z7-14:Ald yielded increased catches of CPC males (Table 2). The catches of male CPC in traps baited with any of the dosages of Z7-14: Ald tested were greater than those in traps baited with virgin females (Table 2).

Monitoring by the Pheromone Trap Figure 2 shows the catches of CPC males in the pheromone-baited traps in pomelo orchards of three different villages in Vinh Long Province in 2007. The numbers of captured males in My Hoa village generally were greater than those in Dong Thanh and Vinh Xuan villages. While adults were caught throughout the year, relatively lower numbers of males were caught from May to September, the rainy season in the Mekong Delta of Vietnam.

Mass Trapping by the Synthetic Pheromone The efficacy of mass trapping on reduction of CPC damage is shown in Table 3. In My Hoa village, over a thousand males were caught in the pheromone-treatment plot. Damage in this plot was less than in the untreated plot, but higher than in the insecticide-treated plot (Table 3a). In Dong Thanh village, in the pheromone-treated plot, fewer males (716) were caught and damage levels were similar to the plot with the insecticide but less than that in the untreated plot (Table 3b).

Disruption of Mating Communication by the Synthetic Pheromone Results of the mating disruption experiments are shown in Table 4. In 2008, pheromone traps in areas treated with dispensers, either at a rate of 200 or 400 dispensers/ha, captured no males over one and half months, while three traps in the untreated orchard (control) captured

Table 1 Catches of *Prays endocarpa* males in a pomelo orchard[a] in traps baited with (*Z*)-7-tetradecenal (Z7-14:Ald), (*Z*)-7-tetradecenyl acetate (Z7-14: OAc), and (*Z*)-7-tetradecen-1-ol (Z7-14:OH)

	Lure components (mg/septum)			Captured males/trap/week [b]
	Z7-14:Ald	Z7-14:OAc	Z7-14:OH	
a) Test 1	0.5	0	0	35.2±16.8a
	0	0.5	0	0.0±0b
	0	0	0.5	0.0±0b
	0.25	0.25	0	0.7±0.5b
	0.25	0	0.25	0.0±0b
	0	0.25	0.25	0.0±0b
	0.17	0.17	0.17	0.0±0b
	0	0	0	1.2±1.3b
b) Test 2	0.5	0	0	9.7±5.9a
	0.45	0.05	0	13.8±7.0a
	0.45	0	0.05	5.3±2.3ab
	0.4	0.05	0.05	3.1±2.1b
	0	0	0	0.0±0b
c) Test 3	1.0	0	0	5.2±2.3ab
	0.9	0.1	0	5.8±2.3a
	0.7	0.3	0	2.1±0.7bc
	0.5	0.5	0	0.8±0.2c
	0.3	0.7	0	0.1±0.2c
	0.1	0.9	0	0.3±0.7c
	0	0	0	0.0±0c

[a] Tests conducted in My Hoa village, using three replicates of each lure per test. Test 1 from November 24 to December 7, 2009 (F=12.4, DF=23), Test 2 from December 7, 2006 to January 4, 2007 (F=19.7, DF=14, and Test 3 from April 8 to May 6, 2008 (F=5.5, Df=20)

[b] Mean ± SE. Values within each test followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer Test

Table 2 Trap catches of male *Prays endocarpa* testing different dosages of synthetic (Z)-7-tetradecenal. For comparison, a treatment containing three virgin female *P. endocarpa* was included[a]

Treatment	Captured males/trap/week [b]
0.1 mg/septum	9.3±1.4c
0.3 mg/septum	21.5±6.4b
0.5 mg/septum	15.5±6.9b
1.0 mg/septum	39.5±22.1a
0 mg/septum	0.0±0d
Three virgin females	1.4±0.9d

[a] Conducted in a pomelo orchard in My Hoa village from February 6 to March 8, 2007, using three traps for each lure (F=131.1, DF=17)

[b] Mean ± SE. Values followed by a different letter are significantly different at P<0.05 by Tukey-Kramer Test

a total of 211 males, indicating 100% disruption of male catch (Table 4a). A similar result was observed in 2009 (Table 4b), with only one and two males caught in the plots treated with the dispensers at 400 tubes/ha and 200 tubes/ha, respectively, over two and half months, compared to the 291 males caught in the untreated plot. The number of fruit damaged by CPC larvae was also reduced in pheromone-treated orchards. In 2008, about one half of the fruit examined in the control plot was damaged, compared to roughly only 3% in the center of the treated plots (for both densities of dispensers). A reduction in damage also was observed at the periphery of the pheromone-treated plots (Table 4a). While damage generally was lower in 2009 (compared to 2008) in the control area, damage again was lower at both the center and periphery of the pheromone-treated plots (Table 4b). In the pesticide-treated plot (in 2009), fewer males were caught in pheromone traps than in the untreated plots. Damaged fruit occurred at a similar frequency to that found in the untreated plot, and at a greater frequency than in the pheromone-treated plots.

Discussion

Chemical analysis of the pheromone gland of female CPC showed Z7-14:Ald, Z7-14:OAc (tentatively identified), and Z7-14:OH. In field trials, synthetic Z7-14:Ald alone attracted male moths. No synergistic effect of the other two compounds was demonstrated, although increasing proportions of Z7-14:OAc in a two-component blend (with concomitant decreases in the dosage of Z7-14:Ald) resulted in decreased trap catches. Z7-14:Ald previously has been identified as a pheromone component of four species of moths (Ando, 2010; El-Sayed, 2010), including three in the genus *Prays*, the citrus flower moths (*P. citri* and *P. nephelomima*) and the olive moth (*P. oleae*). CPC differs from these congeners morphologically as well as in host range and feeding behavior. All four *Prays* species appear to be attracted to Z7-14:Ald. However, pheromone traps baited with Z7-14:Ald caught only CPC males in our field tests, suggesting that only CPC is found in the Mekong Delta. We will investigate whether CPC is sympatric with *P. citri* and *P. nephelomima* in other areas of Vietnam.

Field evaluations of synthetic pheromone found that a rubber septum loaded with 1.0 mg of Z7-14:Ald was an

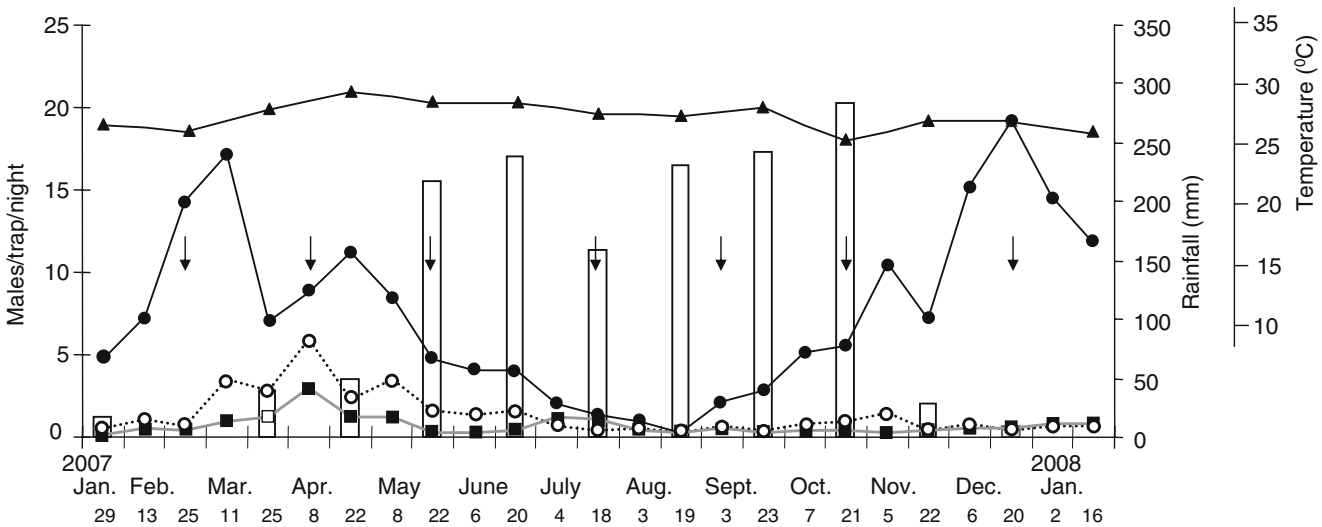


Fig. 2 Pheromone trap monitoring of male moths of the citrus pock caterpillar, *Prays endocarpa*, in three villages of Vinh Long Province in 2007. Three traps were placed in each pomelo orchard and checked approximately every second week on the indicated dates. Males captured by traps in My Hoa village (*black circle*), Dong Thanh village (*white circle*), and Vinh Xuan village (*black square*) are shown. Monthly average temperature, monthly total rainfall, and renewal days of pheromone lures are indicated by *black triangle*, *open bars*, and *arrows*, respectively

Table 3 Percentage of pomelo fruit damaged by *Prays endocarpa* larvae in orchards, containing pheromone traps, without pheromone traps (untreated), or treated with an insecticide[a]

Treatment	Area (ha)	Total fruit examined	Damaged fruit (%)[b]	Total males trapped
a) My Hoa village				
Pheromone trap[c]	0.1	392	25.1±2.0b	1088
Insecticide[d]	0.1	374	11.2±1.4c	–
Untreated	0.1	420	53.3±2.6a	–
b) Dong Thanh village				
Pheromone trap[c]	0.1	469	8.0±1.1b	716
Insecticide[d]	0.1	403	11.4±1.5b	–
Untreated	0.1	398	31.0±8.1a	–

[a] Experiments were carried out in Vinh Long Province from April 4 to June 10, 2008. Damaged fruit were counted on May 15, May 25, and June 10. In (a), F=330.1, DF=9; in (b), F=20.1, DF=8

[b] Mean ± SE. Values within each test followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer Test

[c] Each pheromone treatment used 20 traps baited with synthetic (*Z*)-7-tetradecenal (1 mg/septum). Each lure was renewed monthly and trapped males were counted weekly. Mean trap captures were 54.4±25.4 and 35.8±18.0 of *Prays* sp. in My Hoa and Dong Thanh, respectively

[d] A pyrethroid insecticide (cyhalothrin, Karate 2.5EC) was sprayed three times during the trial

effective tool for monitoring CPC. Our monitoring with pheromone traps showed that adult populations of CPC were high during the dry season and in the early and latter parts of the rainy season. These periods are coincident with the main fruit-bearing stages of pomelo in the Mekong Delta of Vietnam, from February to May, and from September to November. Additionally, CPC males were captured in greater numbers in My Hoa village, than in the two other villages of Dong Thanh and Vinh Xuan. The farming system in My Hoa is a monoculture of pomelo, while in Dong Thanh and Vinh Xuan, pomelo is only one of a number of crops planted, including other fruits, rice, and vegetables. Together, these data suggest that the population density of CPC may be governed by the phenology and density of pomelo fruit.

After identifying an attractive pheromone component, we utilized synthetic pheromone for suppression of damage by CPC in pomelo orchards. In lemon orchards in Israel, pheromone-based mass trapping at a density of 120 traps/ha successfully controlled the citrus flower moth, *P. citri*

Table 4 Pheromone trap catches of male *Prays endocarpa* and percentage of pomelo fruit damaged by *P. endocarpa* larvae in mating disruption experiments in pomelo orchards[a]

Dispenser (Number / ha)	Area (ha)	Total males trapped[c]	Disruption (%)[d]	Total fruit examined	Damaged fruit (%)[b]	
					Periphery	Centre
a) August 15 to September 30, 2008						
400	0.75	0	100	256	9.5±15.0b	3.4±8.1b
200	0.60	0	100	324	14.9±13.3b	2.5±5.3b
0 (no insecticides, control)	0.50	211	–	258	56.1±32.0a	55.4±30.9a
b) August 15 to October 30, 2009						
400	1.1	1	99.6	1637	6.6±0.2bc	3.0±1.6b
200	0.8	2	99.3	2302	4.9±0.9c	4.0±2.2b
0 (no insecticides, control)	0.3	291	–	1525	12.4±4.0ab	15.4±3.2a
0 (insecticide spray)[e]	0.6	63	–	1157	14.2±5.6a	14.6±3.3a

[a] Experiments carried out in My Hoa village

[b] Mean ± SE. Values within each test followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer Test

[c] Three traps baited with synthetic (Z)-7-tetradecenal (1.0 mg/septum) were set on the first day of the experiments in each area. Each lure was renewed monthly and trapped males were counted biweekly for the 2008 trial and weekly for the 2009 trial

[d] Disruption (%) was calculated as $(N_c - N_t)/N_c \times 100$, where N_c and N_t are the numbers of males trapped in a control orchard and an area treated with dispensers

[e] Pyrethroid insecticide (cyhalothrin, Karate 2.5EC) was sprayed three times during the trial

(Sternlicht et al., 1990). In our work, mass trapping of CPC in pomelo orchards at a density of 200 traps/ha resulted in decreased damage to fruit, although the efficacy of this appeared to be dependent upon population density of CPC. This approach could be useful in areas with low densities of CPC damage. In addition to mass trapping, we also attempted mating disruption using polyethylene tubes filled with synthetic Z7-14:Ald, similar to that used for mating disruption of several leaf-roller moths in tea and apple orchards in Japan (Wakamura, 1992; Ando et al., 2004). Mating disruption has been used successfully for the control of a number of moth pests of crop fields, orchards, and forests (Cardé and Minks, 1995), including *P. oleae* in Greece and Egypt (Mazomenos et al., 1999; Hegazi et al., 2009). We found that both densities of tubes used (200 and 400/ha) resulted in virtually complete suppression of trap capture of male CPC. Moreover, the number of fruit damaged by larvae decreased, even in areas treated at 200 tubes/ha, indicating the efficacy of this approach for control of CPC.

This work is a first step in establishing new, sustainable Integrated Pest Management programs in Vietnam. In further work, we plan to establish the minimum density and longevity of dispensers for effective control of CPC throughout the year.

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RAPID COMMUNICATION

Constraints of Simultaneous Resistance to a Fungal Pathogen and an Insect Herbivore in Lima Bean (*Phaseolus lunatus* L.)

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Abstract The existence of tradeoffs among plant defenses is commonly accepted, however, actual evidence for these tradeoffs is scarce. In this study, I analyzed effects of different direct defenses of wild lima bean plants (*Phaseolus lunatus*) that were simultaneously exposed to a fungal pathogen (*Colletotrichum lindemuthianum*) and an insect herbivore, the Mexican bean beetle (*Epilachna varivestis*). Although plants were derived from spatially widely separated populations, I observed a common tradeoff between resistance to pathogens and herbivores. Plants with high levels of anti-herbivore defense (cyanogenesis) showed low levels of resistance to pathogens (polyphenol oxidase activity and phenolic compounds), and *vice versa*. Competition for resources generally is considered to be the basis for tradeoffs. However, I report direct inhibition of polyphenol oxidase by cyanide, making simultaneous expression of both defenses at high levels impossible. I argue that populations composed of individuals investing in one type of defense have an advantage in environments that periodically favor either pathogen or herbivore plant antagonists.

Key Words Tradeoff · Plant defense · *Colletotrichum lindemuthianum* · *Epilachna varivestis* · *Phaseolus lunatus* · Defense syndrome · Cyanogenesis · Polyphenol oxidase · Phenolics

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Introduction

In their natural environment, plants are exposed to multiple antagonists simultaneously, including pathogens as well as herbivores. Consistent with the broad range of attackers, plants generally do not rely on a single defense mechanism, but express multiple defenses (Walling 2000). Theory predicts that constraints on resource allocation produce negative genetic correlations (tradeoffs) between individual traits (Agrawal et al., 2010). In theory, tradeoffs occur where multiple traits are favored by natural selection and compete for shared resources (Thaler et al., 1999), but in practice tradeoffs are rarely found (Agrawal et al., 2010). Such discrepancies between the theory and evidence are difficult to explain without a mechanistic understanding of how tradeoffs affect plant interactions in ecological networks.

Tradeoffs may reflect physiological interference among defensive traits instead of direct resource competition. In lima bean for example, extensive cyanogenesis (anti-herbivore defense) inhibits the activity of polyphenol oxidases (PPO), which are mainly targeted against pathogens. Cyanide binds to the metal-containing active site of PPO (Lieberei et al., 1989). Cyanide-resistant defensive enzymes such as lipoxygenases (LOX) have been demonstrated in some plant species (Tscharntke et al., 2001), but PPOs in lima bean are cyanide-sensitive (Ballhorn et al., 2010a).

I asked how two traits that act against different types of antagonists (Ballhorn et al., 2010a,b) functionally interact when lima bean plants are under simultaneous attack by fungal pathogens and insect herbivores. Under experimental greenhouse conditions, I used wild lima bean plants derived from three different populations in North America, Central America, and the Caribbean to test the concerted effects of cyanogenesis and PPO activity on resistance to a fungal bean pathogen (*Colletotrichum lindemuthianum*) and

a specialist insect herbivore, the Mexican bean beetle (*Epilachna varivestis*).

Methods and Materials

Plants Lima bean plants were grown from seeds collected at natural sites from a Mexican (near Puerto Escondido, Pacific coast of Oaxaca 15°55′31.80″N, 97°9′4.68″W, 8 m a.s.l.), a Costa Rican (near Asserri, San Jose 9°51′48.02″N, 84°5′42.07″W, 1900 m a.s.l.), and a Cuban (Montecristo, Guantanamo 20°7′12.15″N, 74°14′58.01″W, 168 m a.s.l.) population. Plants were cultivated under greenhouse conditions as described in Ballhorn et al., (2007) for 5 wk prior the experiments.

Pathogens and Insects The anthracnose-causing fungal bean pathogen *Colletotrichum lindemuthianum* (strain 12250 purchased from the DSMZ, Braunschweig, Germany), was maintained on oat flake medium (30 g oat flakes, 15 g agar, 1000 ml H_2O) according to DSMZ instructions. To induce sporulation, sterile young snap bean pods (*Phaseolus vulgaris*) were inoculated with a suspension of *C. lindemuthianum* mycelia and incubated at 22±1°C for 14 d in the dark. From these cultures, conidial suspensions were prepared by filtering the homogenate through 3 cm cotton in a 60 ml syringe to remove mycelial fragments. Spore concentration in pooled solutions was adjusted to 10^5 conidia ml^{-1} with sterile distilled water using an Improved-Double-Neubauer counting-chamber. Mexican bean beetles (MBB) were maintained on snap bean as described in Ballhorn et al., (2010b). Freshly molted third-instar larvae were used for the experiments.

Experimental Setup Twenty-four plants (in blocks of 6×4 plants) from each population were used for the experiment in growth chambers under conditions similar to those used for greenhouse cultivation (Ballhorn et al., 2007). Plants of the same block (= population) had contact with each other, whereas the different blocks did not. At the end of the light period, every plant was sprayed from the lower side with 5 ml spore suspension. On the following day, one MBB larva was placed on each plant. Third-instars are relatively mobile and move between contacting plants searching for suitable hosts but do not leave their food plants completely.

Data Collection and Chemical Analysis After an experimental period of 8 d, the number of fungal lesions was counted, and feeding damage was quantified for every plant. Missing leaf area was quantified with a LICOR LI-3100 Area Meter (Walz GmbH, Effeltrich, Germany). Every leaf was scanned three times, and the mean was used for further analysis. To characterize defensive features of plants, leaf discs from undamaged areas of young leaves (Ballhorn et al., 2010c) were analyzed chemically. Leaf cyanogenic potential was analyzed by complete enzymatic degradation of cyanogenic glycosides in leaf extracts followed by 20 min of incubation at 30°C in gas-tight glass vessels (Thunberg-vessels) and spectrophotometric measurement at 585 nm using the Spectroquant® cyanide test (Merck, Darmstadt, Germany) (Ballhorn et al., 2011). Enzymatic activity of PPO was determined polarographically, using the Clark electrode system (Yellow Springs Instruments, Yellow Springs, OH, USA) to measure O_2−consumption during the oxidation of polyphenols and their derivates to quinones (Ballhorn et al., 2010a). Total phenolics were determined in aqueous acetone extracts of leaves with the Folin method using epicatechin (Sigma, Deisenhofen, Germany) as the standard.

Statistical Analyses One-way ANOVAs were applied to test for significant differences of chemical traits, number of lesions, and consumed leaf area among populations. *Pearson's correlations* were used to test for co-variation of variables. Analyses were carried out with PASW Statistics 18.

Results and Discussion

Lima bean plants varied by a factor of 5.15 between the lowest and highest values measured for cyanogenic potential (HCNp, total amount of cyanogenic precursors in leaf tissue). There also was variation in PPO activity (factor of 16.01), and in concentration of total phenolics (factor of 2.76). As I observed no significant differences of traits among lima bean plants from the different origins (HCNp: $F_{2,69}$=0.670, P=0.515; PPO activity: $F_{2,69}$=0.048, P=0.953; total phenolics: $F_{2,69}$=2.469, P=0.092; according to *one-way ANOVA*), data were pooled for further analyses. Cyanogenic potential of plants was correlated negatively with PPO activity and concentration of total phenolics, whereas PPO activity and phenolics showed a positive correlation (according to *Pearson's correlation*; Fig. 1a–c). Consumed leaf area was correlated negatively with HCNp but showed a significantly positive correlation to PPO activity and phenolics (Fig. 1d–f). The number of lesions showed a positive correlation to HCNp (Fig. 1g) but was correlated negatively to PPO activity and concentration of phenolics (Fig. 1h,i). The observed defensive effects of cyanide on the insect herbivore as well as of PPO activity on the fungal pathogen are in line with previous studies (Gleadow and Woodrow 2002; Ballhorn et al., 2010a,b). However, the positive correlation between consumed leaf area and PPO activity (Fig. 1e) or total phenolics (Fig. 1f) is more likely due to negative effects of HCN on insects than to

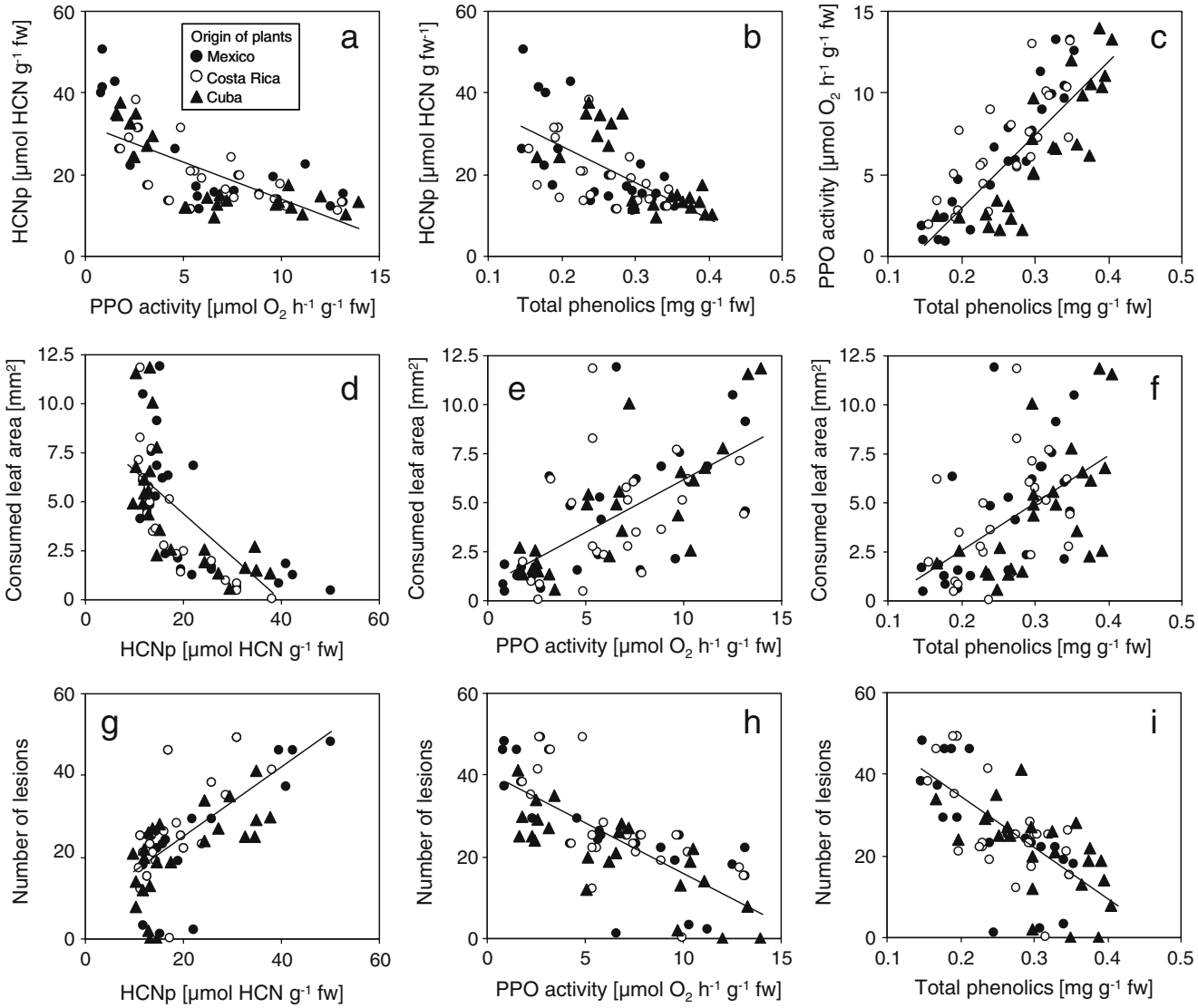


Fig. 1 Correlations between plant traits and plant resistance to fungal and herbivore antagonists. Lima bean plants from three different populations (Mexico, Costa Rica, and Cuba) were analyzed for cyanogenic potential (HCNp; total amount of cyanogenic precursors in leaf tissue), polyphenol oxidase (PPO) activity and concentration of total phenolics. In experiments in which plants were simultaneously exposed to a fungal pathogen (*Colletotrichum lindemuthianum*) and a specialist insect herbivore (Mexican bean beetle; *Epilachna varivestis*), damage caused by each plant antagonist was quantified. To search for potential tradeoffs among plant traits and resistance to fungal pathogens and insect herbivores, the following *Pearson's correlation* were conducted (**a**: $r=-0.716$, $P<0.001$; **b**: $r=-0.604$, $P<0.001$; **c**: $r=0.710$, $P<0.001$; **d**: $r=-0.682$, $P<0.001$; **e**: $r=0.646$, $P<0.001$; **f**: $r=0.534$, $P<0.001$; **g**: $r=0.659$, $P<0.001$; **h**: $r=-0.732$, $P<0.001$; **i**: $r=-0.558$, $P<0.001$)

positive effects of PPOs and phenolics (Ballhorn et al., 2010a). It also is likely that the negative correlations between HCNp and PPO activity as well as between HCNp and phenolic content are the underlying cause of the positive correlation obtained here between the number of pathogen lesions and HCNp (Fig. 1g).The complex interdependence of various chemical plant traits and the resulting effects on different plant antagonists shows that correlations have to be interpreted with care—in particular when only one trait and one corresponding effect is analyzed—as some correlations might not be of functional importance.

The present study on wild lima beans demonstrates that tradeoffs between cyanogenesis and PPO activity as well as cyanogenesis and phenolics occur in natural bean populations distributed over large spatial scales. More importantly, the work shows that these tradeoffs actually result in an ecologically important tradeoff between resistance to pathogens and herbivores when plants were exposed to both antagonists simultaneously. Among all plants, feeding damage and lesion number showed a highly significant negative correlation ($r=-0.600$, $P<0.001$ according to *Pearson's correlation*). From the ecological and population-genetics

perspective, the expression of either anti-fungal or anti-herbivore defenses may be interpreted as an adaptation to variable environmental conditions, which either favor the occurrence of pathogens (humid conditions) or enhance the pressure by herbivores (dry periods) in the natural habitats of lima bean (pers. observation). Thus, variability of defensive traits allows populations to overcome periodically changing environmental conditions not only on the seasonal level but also over longer periods for example due to El Niño climate episodes. In evolutionary terms, periodically changing environmental conditions may represent a stabilizing factor for maintaining this tradeoff between anti-pathogen and anti-herbivore defenses in natural lima bean populations.

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Why Feed on Fungi? The Nutritional Content of Sporocarps Consumed by Buffy-Headed Marmosets, *Callithrix flaviceps* (Primates: Callitrichidae), in Southeastern Brazil

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Abstract The typical diet of *Callithrix* marmosets is based on gums, although fungi are the dietary staple of a single group of *Callithrix flaviceps* studied in the Augusto Ruschi Biological Reserve, in southeastern Brazil. Here, we present the nutritional composition of two species of *Mycocitrus* fungi consumed by *C. flaviceps*, and discuss possible determinants of the preference of the marmosets for fungi over gums. The fungi were high in sugar/fiber and poor in proteins, and were similar in composition to the gums exploited by other marmosets and to the fungi consumed by *Callimico goeldii*. The reduced protein content of the fungi may be offset by the arthropod component of the diet of the *Callithrix flaviceps* study group. The low relative metabolic rate and enlarged cecum of *Callithrix* enable these marmosets to exploit foods with a high fiber content. In addition, the greater digestibility of chitin in comparison with plant fiber may make fungi a nutritionally more valuable resource to marmosets than gums. Marmosets, thus, may prefer to feed on fungi when an adequate supply of this resource is available in the environment.

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Key Words Mycophagy · Atlantic forest · *Mycocitrus* · Diet · Feeding choices · Primate · Callitrichidae

Introduction

Primates exploit a wide range of resources, including reproductive and non-reproductive plant parts and animal prey (Chapman et al., 2002). Despite this, fungi constitute an unusual resource for primates, and mycophagy has been recorded only to date in 23 of the more than 400 recognized species (Hanson et al., 2006; Hilário and Ferrari, 2010). In most cases, fungi are an occasional food used to complement the diet as and when available, although for some species, such as the black snub-nosed monkey (*Rhinopithecus bieti*) and Goeldi's monkey (*Callimico goeldii*), fungi are a dietary staple (Kirkpatrick et al., 1998; Porter, 2001).

Food quality is an important factor influencing foraging decisions (Chapman and Fedigan, 1990). In addition to nutrients such as carbohydrates, proteins, and lipids, which provide essential substances for bodily functions (Maynard and Loosli, 1974), fiber is an important digestive aid (NRC, 2003). While they vary considerably in their composition, fungi tend to be a relatively rich source of carbohydrates, although much of this component may be present in the form of fiber, which requires specializations for efficient digestion (Claridge and Cork, 1994; Claridge et al., 1999).

Atlantic Forest marmosets (genus *Callithrix*) are small-bodied monkeys specialized for the dietary exploitation of plant exudates (gums), which are relatively rich in polysaccharides and minerals such as calcium. Their primary feeding specializations are their chisel-like lower incisors, which allow them to gouge the bark of gum-producing plants to provoke the flow of exudates (Rosenberger, 1978),

and an enlarged cecum, in which the gums are probably fermented for digestion (Coimbra-Filho et al., 1980).

Two cases of mycophagy are known in *Callithrix*, in populations that inhabit montane coastal forests in southeastern Brazil (Corrêa, 1995; Hilário and Ferrari, 2010). In the case of buffy-headed marmosets, *Callithrix flaviceps*, which is reported here, fungi were a staple food, and largely have substituted both gum and fruit in the group's diet. In this research, samples of sporocarps consumed by these marmosets were analyzed to establish their nutritional content for comparisons with other fungi consumed by mammals, and with gums exploited by *Callithrix* marmosets, in an attempt to evaluate the factors that determine the preference for fungi over gum in the *C. flaviceps* study group.

Methods and Materials

Study Site and Group Ecological data were collected at the Augusto Ruschi Biological Reserve (RBAR—19°52′ S, 40°33′W), a 3589 ha fragment of montane Atlantic Forest, located in the Brazilian state of Espírito Santo. The vegetation within the study group's home range varied from primary to early successional forest, with dense stands of *Merostachys* bamboo in many areas. The composition of the diet of a free-ranging group of *C. flaviceps* was estimated by using scan sampling records (Altmann, 1974) collected between January and December, 2008. Data were collected on 6 to 9 days per month, with a 1-min scan being conducted at 5-min intervals throughout the daily activity period on a total of 91 days (Hilário and Ferrari, 2010).

Nutritional Analysis The marmosets consumed two species of *Mycocitrus* (Ascomycetes) fungi, *Mycocitrus* sp. 1 and *Mycocitrus* sp. 2 (Fig. 1), the sporocarps of which were invariably attached to the stems of bamboos of the genus *Merosthachys*. Samples of sporocarps were collected between July and December, 2008. Approximately 200 g of sporocarps of *Mycocitrus* sp. 1 and 130 g of *Mycocitrus* sp. 2 were collected for analysis. Samples initially were dried in a plant specimen drier under an incandescent lamp, triturated in a Wiley mill, sieved through a size 42 mesh, and then stored in tightly-sealed glass flasks at room temperature until processing. The sporocarps were triturated together to produce a single homogeneous sample for each species. Three sub-samples were analyzed for each species, and the results are presented as simple means, given the negligible variation among them. During processing, the samples were first dried in a laboratory oven at 100–105°C, following Cuniff (1995), to determine the humidity level by weighing the material before and after drying. The ash content was determined by heating the material, initially over a Bunsen burner, and then in a muffle furnace at 550°C, until it had turned into lightly-colored ash.

Nitrogen content was determined using the Kjeldahl method and multiplied by a Nitrogen/protein factor of 6.25, following the procedure recommended by Cuniff (1995). Lipid content was determined through hot extraction in ethyl ether, by the Soxhlet method, using QUIMIS-Q-308 G26 as the extractor. An attempt was made to determine the gross fiber content of the sample by acid digestion with citric, acetic, and trichloroacetic acids, as recommended by Diemair (1963), but during processing, the material became gelatinized, possibly due to the presence of complex carbohydrates. As this can compromise the digestion and filtering process, and affect the reliability of the results, we opted to estimate the fiber and sugar content as a single value, by subtracting the weight of the other substances (protein, lipid, and ash) from that of the sample. Caloric value of the fungi, per gram, was calculated by multiplying the proportion of protein by 4.80, carbohydrates by 4.10, and lipids by 9.45, according to Paine (1971).

Results

Diet of the C. flaviceps Study Group During the 12 months of the study period, a total of 2278 records of feeding behavior were collected, and in 1673 of these, the item being ingested was identified. Hilário and Ferrari (2010) proposed that most of the unidentified records probably

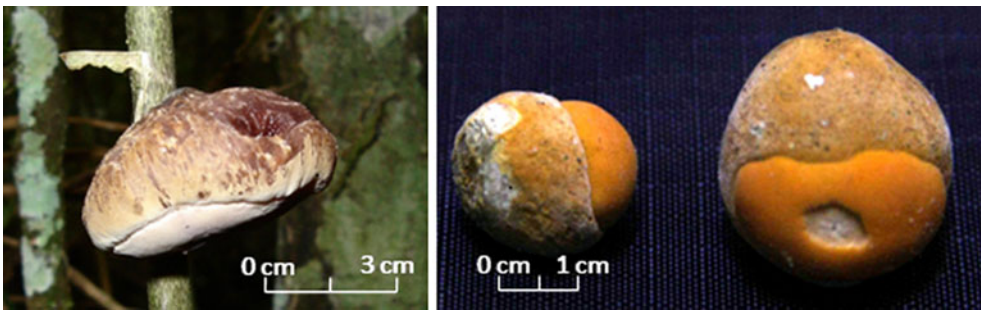


Fig. 1 The two *Mycocitrus* morphospecies exploited by the *Callithrix flaviceps* group in the RBAR, Espírito Santo, Brazil. Photographs: Frederico Pereira. Reproduced with permission from Hilário and Ferrari, 2010

were related to the ingestion of fungi, in which case, the contribution of this item to the diet would have been underestimated. Even so, fungi contributed 64.6% of feeding records for identified items on average over the study period, and never contributed less than 54.0% in any 1 month. In addition to being the most mycophagous diet recorded for any platyrrhine species, including *Callimico goeldii* (Porter, 2001; Porter et al., 2007), the animals appeared to ignore other plant resources (fruit and exudates). In other words, they fed preferentially on fungi, even when the resources typically exploited by other marmosets were available in the environment.

The role of fungi as a dietary staple throughout the year for the *C. flaviceps* study group is comparable to that of plant exudates in the diets of other *Callithrix* marmosets. This includes a second *C. flaviceps* group studied at a site where edible fungi were apparently rare (Ferrari, 1991; Guimarães, 1998).

Nutrient Content of the Sporocarps Moisture represented 11.49±0.03% of the composition of *Mycocitrus* sp. 1 and 9.78±0.03% for *Mycocitrus* sp. 2. These values are not necessarily comparable with those from other studies, however, due primarily to the fact that the sporocarps were partly dried, rather than fresh.

The two species of fungi analyzed here were relatively poor in protein, in comparison with other fungi and most gums (Table 1), although lipid content was similar to that of other fungi. Fiber/sugar content was relatively high in comparison with other fungi, by contrast, and more compatible with that of the gums. The ash content of *Mycocitrus* also was more similar to that of gums than to other fungi.

The two *Mycocitrus* species also were highly similar in their energy content, with 4.18 kcal per gram for species 1, and 4.12 kcal/g for species 2. These values are slightly higher than those obtained for *Auricularia auricula* (4.10 kcal/g) and *A. delicata* (4.01 kcal/g) by applying Paine's (1971) formula to the proportions of nutrients provided by Hanson et al. (2006)—see Table 1. Practically all these values also are within the range for gums—4.02–4.28 kcal/g—provided by Passamani (1996) and Melo et al. (1997).

There are considerable disparities in some values—in particular, the two analyses of *Acacia paniculata*—which may reflect either methodological differences or real variation in the composition of the samples. Despite this variation, one other clear pattern apparent from the data is the greater similarity of the composition of *Mycocitrus* to that of *Ascopolyporus* and *Auricularia* (the bamboo fungi

Table 1 Nutritional content, in dry matter basis, of fungi consumed by several species of mammals, including *Callithrix flaviceps* in the augusto ruschi biological reserve, and gum, consumed by other *Callithrix* species

Resource/species	Fruiting habitus (fungi only)	Content (% dry matter) of:				Reference
		Ash	Proteins	Lipids	Fiber+sugars	
Sporocarp						
Mycocitrus sp. 1	Epigeous	0.96	2.29	1.85	94.89	This study
Mycocitrus sp. 2	Epigeous	1.57	4.60	1.08	93.86	This study
Ascopolyporus polychrous	Epigeous	2.9–3.2	5.5–9.5	0.0–1.6	69.6–78.5	(Hanson et al., 2006)
Auricularia auricula	Epigeous	3.1	10.1	1.4	85.0	(Hanson et al., 2006)
Auricularia delicata	Epigeous	4.0	13.4	0.9	80.0	(Hanson et al., 2006)
Boletus edulis	Hypogeous	1.7	37.5	2.2	29.6[a]	(Bozinovic and Muñoz-Pedreros, 1995)
Elaphomyces granulatus	Hypogeous	3.4–5.4	15.4–23.8	–	26.0–44.8[b]	(Cork and Kenagy, 1989)
Mesophellia glauca	Hypogeous	2.9	10.2	–	46.6[a]	(Claridge and Cork, 1994)
Rhizopogon luteolus	Hypogeous	2.0	10.0	–	18.9[a]	(Claridge and Cork, 1994)
Gum						
Acacia karroo	–	2.3–3.4	0.7–1.1	0.1	75.9–77.6	(Bearder and Martin, 1980)
Acacia paniculata	–	0.8–1.7	2.6–3.5	0.3–0.4	94.5–95.9	(Melo et al., 1997)
	–	2.0	11.4	2.5	84.1	(Passamani, 1996)
Paulinia carpopodia	–	2.0	16.3	2.9	78.7	(Passamani, 1996)
Bauhinia angulosa	–	2.0	6.8	3.1	88.1	(Passamani, 1996)
Dyctioloma incanensis	–	2.0	7.6	3.6	86.7	(Passamani, 1996)

[a] Neutral detergent fiber;

[b] Total cell wall constituents

consumed by *Callimico*) in comparison with the hypogeal species, especially in relation to proteins and fibers/sugars.

Discussion

Overall, the nutritional composition of the fungi consumed by *Callithrix flaviceps* is similar to that of both the fungi consumed by *Callimico goeldii* (Hanson et al., 2006) and to the gums exploited by other *Callithrix* species (Melo et al., 1997; Passamani, 1996—see Table 1). As fungi contributed to the diet of the *C. flaviceps* study group in a proportion similar to that of gum in other *Callithrix* studies (see Hilário and Ferrari, 2010), it seems reasonable to conclude that the fungus-rich diet recorded in the present study had a nutritional balance similar to that of other marmosets.

A small, but potentially important difference is the smaller protein content of the *Mycocitrus* fungi in comparison with gums (Table 1). The values for the fungi may even be overestimated given that they were determined via the nitrogen content, which includes the proportion present in the carbohydrate chitin molecules of the fungus. While the protein content of the fungus may be lower than the daily requirement for marmosets (6.6% dry matter for *Callithrix jacchus*: NRC, 2003), this may not be a limiting factor, given that 25.8% of the study group's diet was made up of animal prey, primarily arthropods, which are an excellent source of protein (Sterling et al., 1994; Baker et al., 1998).

Based on the results of Hanson et al. (2006), it seems reasonable to conclude that the carbohydrates in *Mycocitrus* are composed primarily of fiber, which slows the transit time of food through the gut (Power and Oftedal, 1996), and may thus impose ecological constraints on small-bodied animals such as marmosets (adult body weight 300–400 g), given their relatively higher metabolic rates (Milton, 1984). However, marmosets have a lower metabolic rate than predicted by their body weight (Ross, 1992), and an enlarged cecum, which is thought to aid in the digestion of fiber through bacterial fermentation (Coimbra-Filho et al., 1980).

The fiber of fungi is mainly chitin (Martin, 1979), which is also the primary component of the arthropod exoskeleton. Some insectivorous mammals produce chitinase (Jeuniaux, 1961; Cornelius et al., 1975) and, while this has not been confirmed in marmosets, chitin is more easily digested by these primates than plant fiber (Krombach et al., 1984). In this case, fungi may represent a more nutritionally valuable resource for marmosets than gums, in which the fiber content is composed primarily of heteropolysaccharides, with a predominance of arabinose, galactose, rhamnose, and aldobiouronic acids (Bearder and Martin, 1980). The higher nutritional quality of fungi in comparison with exudates could thus account for the preference of fungi over exudates, and explain why these marmosets appeared to ignore fruit during their foraging activities.

Fungi nevertheless pose other challenges. Edible species are rare in most forests and have slow rates of regeneration (Hanson, 2000; Hanson et al., 2003), which may impose a need for relatively large home range, given the need to ensure access to a number of feeding patches sufficient to maintain an adequate diet over the course of the year. The *C. flaviceps* study group occupied a home range approximately four times larger than the next largest recorded to date for any marmoset group (Hilário, 2009). Relatively large home ranges are also characteristic of a second callitrichid, *Callimico goeldii* (Porter et al., 2007), a characteristic which also has been attributed to the importance of mycophagy in the ecology of this species (Porter and Garber, 2010). In the marmosets, this large home range problem may nevertheless be partly offset by other benefits, such as the avoidance of bark gouging, which is necessary to maintain a regular supply of gum exudates (Digby et al., 2011). Given the findings of the present study, the predominance of gum in the diets of other marmosets may be primarily a consequence of the relative lack of edible fungi at most study sites. Future studies should target the understanding of the relationship between the consumption of fungi and their availability within the environment. Systematic analyses of the nutritional content of other marmoset foods will provide more definitive insights into the factors that determine feeding and foraging choices.

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Variation in Herbivory-induced Volatiles Among Cucumber (*Cucumis sativus* L.) Varieties has Consequences for the Attraction of Carnivorous Natural Enemies

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Abstract In response to herbivory by arthropods, plants emit herbivory-induced volatiles that attract carnivorous enemies of the inducing herbivores. Here, we compared the attractiveness of eight cucumber varieties (*Cucumis sativus* L.) to *Phytoseiulus persimilis* predatory mites after infestation of the plants with herbivorous spider mites (*Tetranychus urticae*) under greenhouse conditions. Attractiveness differed considerably, with the most attractive variety attracting twice as many predators as the least attractive variety. Chemical analysis of the volatiles released by the infested plants revealed significant differences among varieties, both in quantity and quality of the emitted blends. Comparison of the attractiveness of the varieties with the amounts of volatiles emitted indicated that the quality (composition) of the blend is more important for attraction than the amount of volatiles emitted. The amount of (*E*)-β-ocimene, (*E,E*)-TMTT, and two other, yet unidentified compounds correlated positively with the attraction of predatory mites. Quantities of four compounds negatively correlated with carnivore attraction, among them methyl salicylate, which is known to attract the predatory mite *P. persimilis*. The emission of methyl salicylate correlated with an unknown compound that had a negative correlation with carnivore attraction and hence could be masking the attractiveness of methyl salicylate. The results imply that the foraging success of natural enemies of pests can be enhanced by breeding for crop varieties that release specific volatiles.

Key Words Cucumber · *Tetranychus urticae* · *Phytoseiulus persimilis* · Induced plant volatiles · Jasmonic acid · Volatile variability

Introduction

In nature, plants are challenged by a wide range of herbivorous arthropods. Herbivory can severely reduce reproductive success of native plants, and in crops herbivore infestation can result in severe yield losses (Schoonhoven et al., 2005). Plants have developed a multitude of survival strategies, which include constitutive physical and chemical barriers, tolerance mechanisms, and induced defenses that are activated upon attack, such as herbivore-induced plant volatiles (HIPV) that attract natural enemies of the herbivores.

Natural plant odors usually are blends of many different compounds. Upon infestation by herbivores, the composition of the emitted plant volatile blends changes (Arimura et al., 2005). It is widely recognized that these HIPV play an important role in host/prey searching behavior of predators and parasitoids of herbivorous arthropods (Vet and Dicke, 1992; Dicke, 1999; Heil, 2008; Dicke and

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Baldwin, 2010). The release of these attractants also can be achieved by wounding the plants in combination with the application of herbivore regurgitant to the damaged sites or by exposing the plant to phytohormones such as jasmonic acid (JA) (Turlings et al., 1993; Hopke et al., 1994; Dicke et al., 1999; Halitschke et al., 2001).

The two-spotted spider mite, *Tetranychus urticae* Koch, is a common pest of many glasshouse vegetables, including cucumber. *Tetranychus urticae* feeds on individual leaf cells, causing the reduction of total chlorophyll content and net photosynthetic rate of leaves (Bondada et al., 1995; Iatrou et al., 1995). Such leaf cell and tissue injury alters carbon allocation patterns of plant organs (Wyman et al., 1979), often causing deformation of plants (Campbell et al., 1990), and finally often results in a decrease of the productivity of the crop or in death of the plant (Park and Lee, 2005). Previous studies have shown that *T. urticae* infestation or JA treatment induced the emission of volatiles in cucumber plants that attract the predatory mite *Phytoseiulus persimilis* (Bouwmeester et al., 1999; Dicke et al., 1999; Gols et al., 1999; Agrawal et al., 2002; Kappers et al., 2010), a major natural enemy of the spider mites (Sabelis and Dicke, 1985). Predatory mites are blind and rely on odors to locate distant prey patches (Sabelis and Van De Baan, 1983). Arrestment of predatory mites on plants can be influenced not only by the presence of prey, but also by leaf surface characteristics (Krips et al., 1999), and by HIPV (Sabelis et al., 1984).

The quantitative and qualitative HIPV emission varies among plant species (Van Den Boom et al., 2004) and within plant species among varieties (Loughrin et al., 1995; Takabayashi and Dicke, 1996; Krips et al., 2001; Hoballah et al., 2002; Degen et al., 2004; Bukovinszky et al., 2005; Kappers et al., 2010), which may result in differences in attractiveness to the natural enemies of herbivores. However, little to nothing is known about the consequences of these differences among plant genotypes for the effectiveness of the natural enemies under field or greenhouse conditions.

The attractiveness of typical spider mite-induced plant volatiles has been reported for (*E*)-β-ocimene, methyl salicylate, linalool, nerolidol, (*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT], and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E,E*)-TMTT] (Dicke et al., 1990a; De Boer and Dicke, 2004a,b; De Boer et al., 2004; Kappers et al., 2005). Furthermore, results obtained by de Boer and Dicke (2004a) suggest that *P. persimilis* is attracted by methyl salicylate after the mites have been exposed to a methyl salicylate-containing odor blend.

Studies that address the attraction to herbivory-induced plant volatiles have been performed mostly under standardized experimental conditions, such as two-choice Y-tube set-ups (but see De Moraes et al., 1998; Kessler and Baldwin, 2001; Heil, 2004). In this study, we analyzed the attraction of the predatory mite *P. persimilis* to the volatiles of eight cucumber varieties upon spider-mite infestation under realistic greenhouse conditions. In addition, we analyzed the volatile blends emitted by plants of the cucumber varieties in response to spider-mite infestation or JA-treatment, and analyzed the correlation between these volatiles and predatory mite attraction.

Material en Methods

Plant and Mite Material Eight *Cucumis sativus* L. varieties were used. We obtained the varieties from the Centre for Genetic Resources, The Netherlands (CGN19656), and two Dutch Seed Companies (Table 1). Seeds were germinated in vermiculite, and after 7 d transplanted to 6 L pots with potting compost and nutrient solution. Plants were cultivated in a greenhouse at 23±2°C, 60±10% relative humidity (RH), under a natural daylight photoperiod (April–September). Four-wk-old plants with eleven leaves were used for experiments.

The herbivore *Tetranychus urticae* Koch, the two-spotted spider mite, was reared on bean plants (*Phaseolus vulgaris*) in a greenhouse. Predatory mites, *Phytoseiulus persimilis,* were reared on spider-mite infested bean plants.

Predator Attraction Setup Plants were infested with *T. urticae* to induce herbivore feeding under similar conditions as described above. Per cucumber variety, ten healthy plants were placed in a line with approx. 0.45 m between two neighboring plants. The ten plants were enveloped by a gauze cage (3×6×2.5 m). The 3rd plant was infested with approx. 500 spider mites that were distributed equally over the 7th and 8th leaf. After 3 d, the inoculated infested leaves showed visual signs of damage. Spider mites did not move between leaves, i.e., no mites were found on uninoculated leaves. Three days later, 150 predatory mites, *P. persimilis,* were released on plants 5, 6, and 7 in the row, 50 predators on each plant. The number of walking predatory mites and predator eggs that were found on spider-mite infested plants was counted after 48 and 96 hr after the introduction of the

Table 1 Origins of cucumber varieties used in this study

Code	Varieties & Genetic background
A	CGN19656, Land race from Ban, Iran
B	F1 Hybrid, long European type
C	F1 Hybrid, long European type
D	F1 Hybrid
E	F1 Hybrid
F	F1 Hybrid, long European type
G	F1 Hybrid, long European type
H	F1 Hybrid, mini type

predators. All varieties were included six times, randomly distributed over eight experimental runs, with six varieties per run. The six gauze cages were positioned next to each other in a greenhouse with 1 m between two cages. In each run, an accession had different accessions as neighbors. Per accession, the average number of mobile *P. persimilis* adults on spider-mite infested plants (plant nr. 3) was determined for each time point. Furthermore, we counted the number of *P. persimilis* eggs on these plants, as well as the number of spider-mite adults on these plants.

Headspace Collection To analyze the quantity and composition of herbivory-induced cucumber volatiles, dynamic headspace collection was used in the greenhouse setup. In the dynamic sampling procedure, the volatiles were collected on 200 mg Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, MI, USA) in stainless steel cartridges (Markes, Llantrisant, UK). Headspace air was drawn through the cartridges for 1 hr using a portable battery-operated air sampler with a constant flow of 100±5 ml min^{-1} (Ametek / du Pont de Nemours & Co., type Alpha-2, DEHA International, Huizen, The Netherlands).

For comparison of herbivory-induced cucumber volatiles among varieties, we also used the plant hormone jasmonic acid (JA) to standardize the level of induction. Jasmonic acid induces a similar, though not identical, blend of volatiles as spider-mite infestation (Bouwmeester et al., 1999; Dicke et al., 1999; Gols et al., 1999; Bruinsma et al., 2009; Kappers et al., 2010; Snoeren et al., 2010b). Two healthy 3-wk-old plants of each accession were enclosed in a 2.5 L glass jar and sprayed with 1.0 mM (±) jasmonic acid (Sigma-Aldrich) in 0.01% Tween-20. In total, 20 ml were used to spray 16 plants (8 varieties, 2 plants each). Control plants were sprayed with water with 0.01% Tween-20. Dynamic headspace sampling was carried out in a climate room (20±2°C, 70% RH; L8:D16h photoperiod and 150–170 μmol photons m^{-2} s^{-1} PPFD at the level of the plants). Twenty-four hours after JA treatment, just before headspace collection, jars with plants were closed with a Viton-lined glass lid having an inlet and outlet. Inlet air was filtered by passing it through stainless steel cartridges filled with 200 mg Tenax TA. Volatiles were trapped by sucking air through the jar at a rate of 100 ml min^{-1} through a similar cartridge filled with 200 mg Tenax TA. Headspace volatiles for all treatments were collected for a period of 3.5 hr. Fresh weights of shoots were determined immediately after the experiments. On each experimental day, headspace samples of eight varieties were collected simultaneously. For each accession, the headspace was collected and analyzed for three replicate sets of two plants.

Analysis of Headspace Volatiles Headspace samples were analyzed with a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, MA, USA) quadrupole mass spectrometer. Before desorption of the volatiles, the Tenax cartridges were dry-purged with nitrogen at 30 ml min^{-1} for 30 min at ambient temperature to remove water. Volatiles were desorbed from the cartridges by using a thermal desorption system at 250°C for 3 min (Model Ultra Markes Llantrisant, UK) with a helium flow of 30 ml min^{-1}. Analytes were focused at 3°C on an electronically-cooled sorbent trap (Unity, Markes, Llantrisant, UK). Volatiles were transferred in splitless mode to the analytical column (Rtx-5 ms, 30 m, 0.25 mm i.d., 1.0 μm film thickness, Restek, Bellefonte, PA, USA) by rapid heating of the cold trap to 250°C. The GC was held at an initial temperature of 40°C for 3.5 min followed by a linear thermal gradient of 10°C min^{-1} to 280°C, and held for 2.5 min with a column flow of 1 ml min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 45–400 m/z with a scan rate of 3 scans s^{-1}. Compounds were identified by using the deconvolution software AMDIS (version 2.64, NIST, USA) in combination with NIST 98 and Wiley 7th edition spectral libraries and by comparing their retention indices with those from the literature (Adams, 1995). The identification of (*E*)-β-ocimene, (*E,E*)-TMTT, methyl salicylate, and geranyl isovalerate was confirmed by comparison with authentic standards. For quantification, characteristic quantifier ions were selected for each compound. Metalign software (PRI-Rikilt, Wageningen, the Netherlands) was used to align peaks of chromatograms of all samples and to integrate peak areas for the quantifier ions.

Statistics Individual volatiles were analyzed for significant changes among varieties for the treatments using one-way ANOVA followed by a LSD *post-hoc* analysis (SPSS 15.0, Chicago, Il, USA). The emitted quantities of individual volatile components were analyzed for significant differences among plant treatments by using a *t*-test (SPSS 15.0). Varieties were analyzed for significant differences in the attraction of predatory mites using one-way ANOVA followed by a LSD *post-hoc* analysis (SPSS 15.0).

Overall volatile profiles were analyzed by using Principal Component Analysis (GeneMath XT 2.0) after $\log_{10}$ transformation of the data and subtracting the average value of all treatments to normalize the data (Hendriks et al., 2005). The first few principal components (PCs) explain most of the variance in the original data, and we used it to visualize differences of the total volatile profile among varieties.

Multivariate mass spectra reconstruction (MMSR, Plant Research International, Wageningen, The Netherlands) as developed by Tikunov et al. (2005) was used to reduce the MetAlign dataset of 33.800 mass fragments. By using MMSR, clusters of related metabolite fragments that show

similar patterns over different samples can be recognized as so-called centrotypes on the basis of their overlapping patterns throughout the different samples, and the corresponding metabolites can be subsequently identified.

Results

Eight varieties of *C. sativus* were compared for their attractiveness to *P. persimilis* predatory mites after herbivory by *T. urticae* spider mites (Fig. 1). Twenty-one to 40% of the predatory mites that were released at a distance of 90, 135, and 180 cm from the spider-mite infested plants were retrieved on these infested plants within 96 hr, ranging from 30.8±2 predatory mites retrieved on hybrid D to 59.5±3 on landrace A (Fig. 1a). The number of *P. persimilis* eggs retrieved on these plants was lowest on hybrid D (21.3±2 eggs), and highest on landrace A (34.5±3 eggs) (Fig. 1b).

The total emission of volatiles by plants of each accession after damage by spider mites was estimated by summing all peak areas. All varieties, except accession B had significantly higher emission rates of volatiles than the surrounding non-infested cucumber plants (Fig. 2a). Hybrid B had the lowest emission rate of total volatiles after 96 hr of spider-mite infestation, whereas hybrid G had the highest emission rate. Differences in emission rates were not caused by differences in the numbers of spider mites on the infested plants. The number of spider mites retrieved on plants after 96 hr was lowest on landrace A and highest on hybrid H (Fig. 2b). There was no relation between the numbers of spider mites and the total amount of volatiles emitted across the eight varieties.

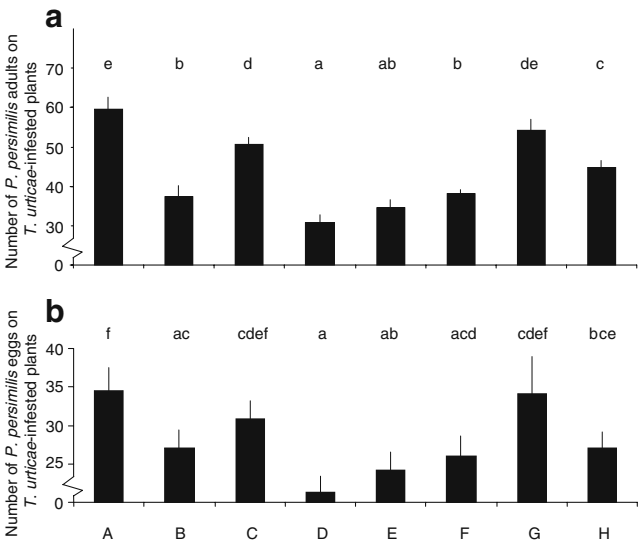


Fig. 1 Mean (± SE) of *Phytoseiulus persimilis* adults (**a**) and eggs (**b**) retrieved on spider-mite infested plants on eight cucumber varieties. *N*= 6 plants. Data were statistically analysed by using a one-way ANOVA followed by LSD *post-hoc*. Varieties followed by different letters indicate a significant difference ($P<0.05$)

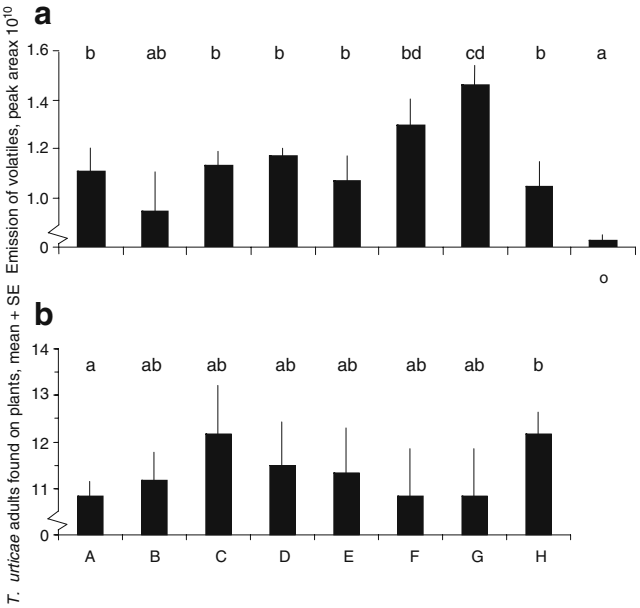


Fig. 2 Total emission of volatiles (mean peak area of all mass fragments ± SE) emitted after 96 hr of spider-mite infestation (**a**) (*N*= 6 plants). Mean number (± SE) of *Tetranychus urticae* adults retrieved on infested plants (**b**) after 96 hr. Different letters indicate significant differences ($P<0.05$) using one-way ANOVA followed by LSD *post-hoc*. The o in Fig. 2a indicates ambient air

To address the question of which volatiles may be responsible for the differences in attractiveness, multivariate exploratory analysis was performed on the headspace compositions by using Principal Component Analysis. The first two principal components of the PCA explained 57% of the variance found in the data set, discriminating the more attractive landrace A and hybrids C, G, and H from the less attractive hybrids B, D, E, and F along vector 1 (47.3%), and the variation among replicates along vector 2 (9.7%) (Fig. 3a). The order of attractiveness of the eight varieties towards predatory mites also is indicated in Fig. 3, with no. 1 representing lowest numbers of predatory mites attracted and no. 8 representing the strongest attraction. This shows that the four most attractive varieties cluster in the left part of the plot, and the four least attractive ones cluster in the right part of the plot (Fig. 3a). The loading plot of the first two principal components is shown in Fig. 3b, and can be used to identify which compounds contribute most to the separation of the varieties in Fig. 3a. However, as hundreds of different volatile compounds can be detected in a headspace sample, quantitative alignment of the different metabolites results in a dataset that comprises almost 34,000 mass fragments (Fig. 3b).

In order to recognize metabolites from those tens of thousands of molecular fragments that constitute a typical GC-MS profile, MMSR was used to assign 363 centro-

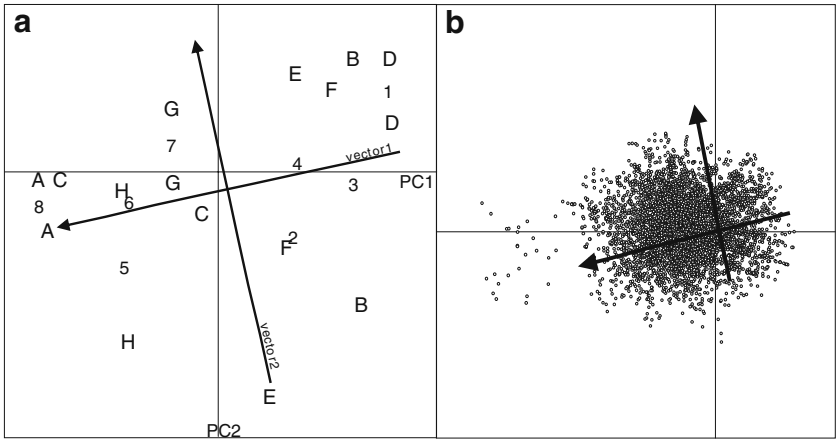


Fig. 3 **a**: Principal Component Analysis of the mass fragments of volatiles emitted by eight cucumber accessions. Accession A (*filled square*, 8), B (*empty square*, 3), C (*empty circle*, 6), D (*filled circle*, 1), E (*empty triangle*, 2), F (*filled triangle*, 4), G (*empty diamond*, 7), H (*filled diamond*, 5). The first PC explains 47.3% of the variation found and the second PC 9.7%. The order of attractiveness of each accession is added along vector 1, with no. 1 representing the accession for which the lowest number of predatory mites was recorded and no. 8 representing the accession for which the highest number of predatory mites was recorded on spider-mite infested plants. The variation between replicates is added along vector 2. **b**: Loading plot with 33.800 mass fragments

types. Pearson correlation coefficients were calculated between the peak areas of each centrotype and the attractiveness data for the whole dataset. In addition, the dataset was subjected to stepwise regression with backward elimination, where the contribution of each predictor was determined one by one by using the *t*-test significance value, and centrotypes that did not contribute to significance were omitted from the model. Supplementary Online

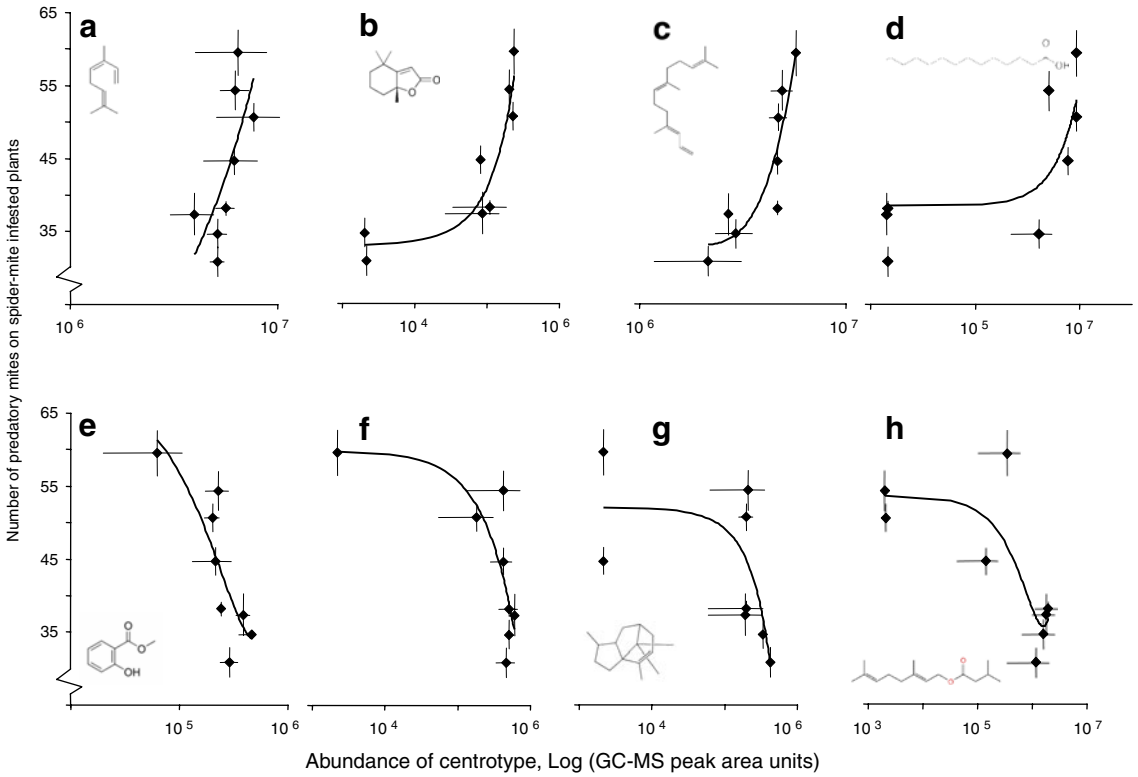


Fig. 4 Correlation of eight selected centrotypes and the number of predatory mites found on each of the varieties included in this study. **a**: Centrotype 9969, (*E*)-β-ocimene, R^2=0.504; **b**: Centrotype 21281, putative dihydoactinidiolide, R^2=0.871; **c**: Centrotype 23191, (*E,E*)-TMTT, R^2=0.830; **d**: Centrotype 26893, putative myristic acid, R^2=0.431; **e**: Centrotype 13780, methyl salicylate, R^2=0.678; **f**: Centrotype 14154, unknown compound, R^2=0.628; **g**: Centrotype 20359, putative α-patchoulene, R^2=0.511; **h**: Centrotype 22546, putative geranyl isovalerate, R^2=0.655

Material Table 1 shows the centrotypes that were assigned as attractive to *P. persimilis*, their putative identity, and statistics (*P*-values of stepwise regression and Pearson coefficients). Fifteen centrotypes were significantly ($P< 0.05$) affecting predator behavior as determined by stepwise regression, either in a positive or negative way. Eight of these centrotypes also had a strong Pearson correlation (>0.5 or <−0.5) with attractiveness, and these are depicted in Fig. 4. Positive correlations were found for (*E*)-β-ocimene (Fig. 4a), (*E,E*)-TMTT (Fig. 4c), and two other centrotypes (Fig. 4b,d). Centrotype 21281 (Fig. 4b) has a retention index of 1489, and its mass spectrum shows homology with that of dihydroactinidiolide (retention index 1483). The mass spectrum of centrotype 26893 depicted in Fig. 4d corresponds well with that of myristic acid, but the retention index (1809) deviates much from the one reported for myristic acid (1720). Pearson correlation analysis showed that the emission of (*E*)-β-ocimene correlated with the emission of 64 other centrotypes. Five of these centrotypes also showed modest positive correlation with predator attraction (Pearson coëfficient between 0.40 and 0.50), among them (*E,E*)-α-farnesene and an unknown sesquiterpenoid (Fig. 5a). There were no centrotypes with a high correlation with both the amount of (*E,E*)-TMTT and predator attraction.

A negative correlation between attractiveness and amount of the compound was recorded for methyl salicylate (Fig. 4e) and three other centrotypes (Fig. 4f–h). The mass spectra and retention indices of two of these centrotypes correspond well with those of α-patchoulene (Fig. 4g) and geranyl isovalerate (Fig. 4h), respectively. The emission of methyl salicylate correlated with that of 11 other centrotypes. Two of these centrotypes also negatively correlated with attractiveness (Pearson coëfficient between −0.30 and −0.40; Fig. 5b).

For direct comparison of variation in herbivory-induced cucumber volatiles, independent of differences in mite feeding damage due to variation in the level of direct defense, plants were treated with JA. As in previous experiments, JA-treated plants emitted higher amounts of volatiles than did non-treated plants (data not shown), with considerable variation among accessions in the relative proportion of individual compounds. Table 2 shows the amounts of individual volatiles emitted 2 d after JA-treatment by plants of the eight varieties included in this study. The total emission of volatiles in JA-treated plants was highest in hybrid C and lowest in hybrid H. Variation in emission rates for individual volatile compounds among varieties varied between 4-fold differences for (*E,E*)-TMTT and up to almost 3000-fold for linalool.

Discussion

Our data show that there is considerable variation among cucumber varieties in HIPV emission rates as well as the attraction of predatory mites under greenhouse conditions. Attraction to and arrestment on spider-mite infested cucumber plants by predatory mites can be influenced by prey density (Toyoshima and Amano, 1998), leaf surface characteristics (Krips et al., 1999), and HIPV (Sabelis et al., 1984). We assume that, just as was shown for Lima bean plants (Gols et al., 2003), within a cucumber variety the attraction of *P. persimilis* is positively related to the amount of spider-mite damage on cucumber leaves. Hence, among plants of the same variety the total amount of damaged leaf area affects the degree of attraction of the predators. However, across varieties, no significant correlation was found between the number of spider mites and the number of *P. persimilis* retrieved (Pearson correlation coefficient

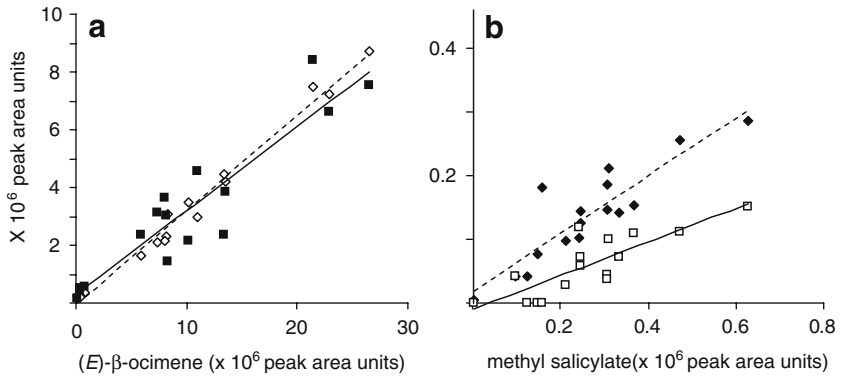


Fig. 5 Correlation between the amounts of selected centrotypes. **a**: between (*E*)-β-ocimene and two other centrotypes, i.e., putative (*E,E*)-α-farnesene (closed squares, solid line, Pearson coefficient with (*E*)-β-ocimene 0.674, Pearson coefficient with attraction of *Phytoseiulus persimilis* 0.384) and an unknown sesquiterpenoid (open diamonds, dotted line, Pearson coefficient with (*E*)-β-ocimene 0.878, Pearson coefficient with attraction of *P. persimilis* 0.441); **b**: between methyl salicylate and two other unknown centrotypes (Pearson coefficient with methyl salicylate 0.640 for closed diamonds and 0.782 for open squares, respectively, Pearson coefficient with attraction of *P. persimilis* −0.421 and −0.346, respectively)

Table 2 Comparison of volatile compounds emitted from JA-treated plants of various cucumber varieties. Amounts emitted are denoted as GCMS area units. Different letters indicate significant differences (α=0.05, LSD post-hoc analysis) between varieties for each component

Compound	Varieties							
	A	B	C	D	E	F	G	H
(*Z*)-3-Hexen-1-ol	7.9×10^{4} a	8.9×10^{4} a	8.5×10^{4} a	7.5×10^{4} a	3.3×10^{5} b	7.4×10^{4} a	9.5×10^{3} c	9.0×10^{3} c
Oxime[a]	1.3×10^{6} a	1.9×10^{6} a	1.8×10^{8} b	9.7×10^{5} a	1.7×10^{7} c	1.9×10^{7} c	2.6×10^{6} a	6.8×10^{5} a
(*Z*)-3-HeAc[b]	1.7×10^{6} a	3.7×10^{6} b	3.6×10^{5} c	4.0×10^{5} c	2.6×10^{6} ab	1.7×10^{6} a	3.2×10^{5} c	1.0×10^{5} c
(*Z*)-β-Ocimene	5.1×10^{5} a	1.7×10^{5} ab	1.8×10^{6} a	1.7×10^{4} b	5.8×10^{6} c	1.9×10^{5} ab	1.3×10^{5} ab	7.5×10^{3} b
(*E*)-β-Ocimene	1.8×10^{6} a	9.3×10^{5} a	4.8×10^{6} b	1.5×10^{5} c	4.4×10^{6} b	5.8×10^{4} c	7.8×10^{5} a	3.4×10^{5} ac
Linalool	2.3×10^{2} a	3.2×10^{1} b	2.6×10^{1} b	4.3×10^{-1} c	2.8×10^{1} b	4.2×10^{1} b	6.2×10^{-1} c	8.2×10^{-2} c
(*E*)-DMNT	8.6×10^{5} a	5.1×10^{5} a	7.9×10^{5} a	8.1×10^{4} b	7.1×10^{6} c	4.7×10^{5} a	2.5×10^{5} ab	1.7×10^{5} ab
Methyl Salicylate	8.1×10^{3} a	1.0×10^{5} b	5.8×10^{4} b	7.2×10^{4} b	3.4×10^{4} ab	8.6×10^{4} b	7.4×10^{4} b	2.6×10^{5} c
(*E,E*)-α-Farnesene	7.6×10^{6} a	2.1×10^{6} ab	5.2×10^{6} a	6.2×10^{4} b	1.4×10^{7} c	4.2×10^{6} ab	1.7×10^{5} c	4.9×10^{5} c
(*E,E*)-TMTT	2.1×10^{5} a	5.2×10^{4} b	5.2×10^{4} b	8.1×10^{4} ab	1.0×10^{5} ab	1.3×10^{5} ab	1.1×10^{5} ab	1.9×10^{5} a
Total[c]	5.2×10^{7} ac	3.9×10^{7} ac	8.9×10^{8} b	7.3×10^{6} a	8.9×10^{7} c	5.6×10^{7} ac	7.3×10^{6} a	7.1×10^{6} a

[a] 3-methyl-butanal-O-methyl oxime

[b] (*Z*)-3-hexen-1-yl acetate

[c] Total emission is estimated by adding all peak areas from the chromatogram

−0.12). Thus, the difference in attraction of predatory mites among varieties was not dependent on the amount of damage. Furthermore, the relative attractiveness of the cucumber varieties to the predatory mites did not correlate with the total amount of volatiles emitted by the spider-mite infested plants of the different varieties (Pearson coefficient 0.29). For example, plants of hybrid G acted as the "loudmouth" in terms of HIPV emission and were very effective in recruiting natural enemies, while hybrid G had the lowest infestation of spider mites compared with the other accessions in this study. In contrast, hybrid D showed a moderate spider-mite infestation and had the third highest HIPV emission, but was not effective in attracting predatory mites in comparison with the other accessions. Thus, when comparing different varieties, it is not just a matter of more damage inflicted by more mites, resulting in higher amounts of volatiles that attract more predatory mites. Rather, certain compounds within the total blend are differentially important for attraction or repellence, and determine total attractiveness of the plants of different varieties to the predatory mites.

To investigate which compounds within the volatile blend correlated to the differences in attraction, we analyzed the headspace of spider-mite-infested cucumber plants under realistic greenhouse conditions and in parallel attractiveness to predatory mites. This implies that headspace was captured at relatively 'dirty' ambient air with a high humidity, which resulted in relatively overcrowded GCMS chromatograms. To avoid technical problems, our GCMS method included dry purging at room temperature and focusing of the samples at temperatures above 0°C, which may have resulted in some loss of highly volatile compounds, such as green leaf aldehydes (Ruther, 2000). We chose to use an untargeted statistical approach to handle the complex GCMS profiles. Using MMSR (Tikunov et al., 2005), more than 360 centrotypes were recognized as metabolites from the tens of thousands of mass fragments, that constitute a typical GCMS profile. No attempt was made to identify all these centrotypes. Instead, we focused on those that had a high Pearson correlation with attractiveness, resulting in a selection of 15 centrotypes that we identified tentatively.

The lowest number of spider mites was retrieved on plants of accessions A, F, and G. Accession A is a land race, and may have enhanced levels of cucurbitacin C compared with the other accessions, which are all hybrids that were selected for non-bitterness. Thus, the level of direct defense could perhaps explain the lower number of spider mites in accession A, although accessions F and G have comparably low numbers of spider mites. Accession A is, however, quite interesting: a low level of herbivory results in the highest attraction of predatory mites, while the amount of HIPV is rather moderate compared to the other accessions. When infested with spider mites, accession A appears to be very sensitive: low numbers of herbivores trigger the induced volatile production cascade in such a way that it results in a volatile blend that is most attractive to the predatory mites and relatively rich in (*E*)-β-ocimene,

(*E,E*)-TMTT, and two other centrotypes that show homology in mass spectrum with dihydroactinidiolide and myristic acid. Furthermore, the volatile blend of accession A contains relatively low levels of methyl salicylate and geranyl isovalerate, and two other repellent centrotypes, of which one shows homology with α-patchoulene. It would be interesting to address how this is regulated genetically and biochemically.

Geranyl isovalerate, dihydroactinidiolide, α-patchoulene, and a putative fatty acid have not been reported previously to be present in the volatile blends of cucumber or other *Cucumis* species (Takabayashi et al., 1994; Agrawal et al., 2002). Dihydroactinidiolide ($C_{11}H_{16}O_2$) occurs naturally in fenugreek (*Trigonella foenum-graecum*), mangos (*Mangifera indica*), and silver vine (*Actinidia polygama*) (Mebazaaa et al., 2009). α-Patchoulene has been reported in *Hypericum* spp., and geranyl isovalerate has been reported to be present in the blend of many plant species (DNP, 2009). The occurrence of myristic acid (or, based on the retention index, probably another fatty acid) has not been previously reported in volatile blends of cucumber, but could likely be present in its blend, because with proceeding spider-mite damage lipoxygenase activity increases and the breakdown of fatty acids occurs.

The responses of carnivores to volatiles have been tested mostly against a minimal odor background in laboratory or simplified greenhouse setups. Several spider-mite induced plant volatiles attract *P. persimilis*, among them linalool (Dicke et al., 1990a), (*E*)-β-ocimene (Dicke et al., 1990a; Krips et al., 2001), (*E*)-DMNT, nerolidol (Dicke et al., 1990a; Kappers et al., 2005), and methyl salicylate (Dicke et al., 1990a; De Boer and Dicke, 2004a,b). Furthermore, (De Boer et al., 2004) reported that, although (*E,E*)-TMTT itself was not attractive to *P. persimilis*, the presence of this compound in the total volatile blend did improve preferences of the predatory mites. Moreover, Van Wijk et al. (2008) suggest that *P. persimilis* does not have an innate preference for (individual) HIPV, but is able to adjust its olfactory response based on experience. An innate preference for specific compounds in combination with the ability to identify these compounds in unfamiliar mixtures could explain how *P. persimilis* prefers, without prior experience, the odor of spider mite-infested plants over that of uninfested conspecifics for several plant species (Van den Boom et al., 2002). We repeatedly found a stronger attraction of predatory mites to cucumber varieties that have higher proportions of (*E*)-β-ocimene, (*E,E*)-TMTT, and putative dihydroactinidiolide and myristic acid in their induced blend, both in laboratory olfactometer experiments (Kappers et al., 2010) as well as in the greenhouse experiments presented here. The predatory mites used in this study were reared on spider-mite infested *Phaseolus vulgaris* plants that are known to emit (*E*)-β-ocimene, (*E*)-DMNT, (*E,E*)-TMTT, and methyl salicylate (Dicke et al., 1990a, 1999). We expect that predatory mites have learned to associate these volatiles with prey.

However, three other compounds have opposite effects: varieties with high proportions of methyl salicylate, geranyl isovalerate, or putative α-patchoulene in their odor profile were less attractive to predatory mites. The negative effect of methyl salicylate on the attraction of predatory mites was unexpected, as this compound is commonly induced after herbivory in many plant species (e.g., Dicke et al., 1990b; Ament et al., 2004; De Boer et al., 2004; Bukovinszky et al., 2005) and has been reported to be an attractive compound to predatory mites (Dicke et al., 1990a; Ament et al., 2004; De Boer and Dicke, 2004a,b). However, in our study, the negative association of methyl salicylate with attractiveness was found within the total HIPV-blend of different cucumber accessions. The presence of methyl salicylate correlated positively with the presence of 11 other centrotypes in the HIPV-blend. Two of these centrotypes also were negatively associated with the attraction of predatory mites. This could imply that one or both of these (unknown) compounds are produced coordinately with methyl salicylate, are repellent towards the predatory mites, and may affect or override the attractiveness of methyl salicylate in the headspace. A repellent effect of methyl salicylate on carnivores also was found for *Diadegma semiclausum*, a parasitoid wasp that preferred the HIPV blend of transgenic *Arabidopsis* lacking methyl salicylate (Snoeren et al., 2010a). Examples of compounds in the HIPV-blend of cucumber with suggested repellent effects to predatory mites are volatile oximes (Takabayashi et al., 1994). Indeed, Agrawal et al. (2002) showed that sweet cucumber plants attracted more predatory mites than bitter, cucurbitacin-containing cucumber plants although the latter plants emitted more volatiles, among them (*E*)-β-ocimene and (*E,E*)-α-farnesene. The explanation for this discrepancy was found in the presence of oximes in the HIPV blend of bitter cucumber plants (Agrawal et al. 2002). Breeding and selection of crops may provide varieties that emit higher amounts of unwanted compounds that have negative effects on attraction of natural enemies. A previous study showed that sweet cucumber plants, lacking cucurbitacins, attracted more predatory mites than plants from bitter varieties that produce constitutive and inducible cucurbitacins. The bitter plants emitted larger amounts of several volatiles that are known attractants of the predatory mite *P. persimilis*, i.e., (*E*)-β-ocimene, (*E*)-DMNT, and methyl salicylate (Agrawal et al., 2002). This also suggests the presence of repellent compounds.

Other examples of repellent compounds present in herbivore-induced plant volatiles blends can be found in systems involving parasitoid wasps. For example, a HIPV fraction of maize containing (*Z*)-3-hexen-1-yl acetate,

linalool, and (*E*)-DMNT was the least attractive compared to other tested fractions of the HIPV blend of maize to the parasitoid *Cotesia marginiventris*; this fraction might even have a repellent or masking effect on other fractions of the HIPV blend (Turlings and Fritzsche, 1999). In addition, isoprenoids also were found to interfere with host finding, as was demonstrated for transgenic *Arabidopsis* plants emitting isoprene (Loivamäki et al., 2008). The function of emitting HIPV that do not lure naïve carnivores but rather repel them may be linked to the compounds' role in other plant defense mechanisms. For example, HIPV that may be involved in microbial resistance can act as repellents towards herbivores (Bruce et al., 2008), which has been demonstrated for methyl salicylate (Snoeren et al., 2010a). However, it is well known that carnivores can learn to respond to HIPV blends, and this also has been demonstrated for the response of predatory mites to prey-associated odor mixtures.

The typical spider-mite induced volatile (*E*)-DMNT (Dicke et al., 1990a; Mercke et al., 2004; Van Den Boom et al., 2004) was emitted by all JA-treated accessions with a 100-fold variation in the range of emission rates (Table 2). Furthermore, linalool was emitted by all accessions in small amounts. No correlation between attractiveness to *P. persimilis* and these HIPV was found in our spider-mite induced plant experiment, although these compounds have been reported for their capacity to attract predatory mites (Dicke et al., 1990a,b; Kappers et al., 2005). In contrast, (De Boer et al., 2004) and (Van Wijk et al., 2008) did not find an attractive effect of (*E*)-DMNT. Linalool was not attractive either in the study by Van Wijk et al. (2008). Our results also indicate that these compounds are not of high importance for prey searching of predatory mites.

All accessions in this study emitted (*E*)- and (*Z*)-β-ocimene. Most accessions have an (*E*)-β-ocimene proportion of the total ocimene amount of more than 70%, except hybrids E and F that have an (*E*)-β-ocimene proportion of less than 50%, and thus a relatively high proportion of (*Z*)-β-ocimene in their blend. Dicke et al. (1990a) reported that (*E*)-β-ocimene is attractive to predatory mites. These authors also assessed the effect of mixtures of (*E*)- and (*Z*)-β-ocimene, and found that only (*E*)-β-ocimene was attractive. However, the response to (*E*)-β-ocimene was attenuated by the addition of (*Z*)-β-ocimene. A racemic mixture of 70% (*E*)-β-ocimene and 30% (*Z*)-β-ocimene was tested by Dicke et al. (1990a) and Van Wijk et al. (2008), and in both studies this blend was not attractive. In contrast, a racemic mixture of 90% (*E*)-β-ocimene and 10% (*Z*)-β-ocimene did attract *P. persimilis* (Dicke et al., 1990a). Thus, the relatively low degree of attraction of the predatory mites to hybrids E and F may be explained (partly) by the relatively high proportion of (*Z*)-β-ocimene in these accessions. Although hybrids C and G do not emit high amounts of (*E*)-β-ocimene in absolute terms, the (*E*)/(Z) ratio is quite high, which agrees with their ranking in attractiveness.

Finally, the emission of (*E*)-β-ocimene is highly correlated with that of (*E,E*)-α-farnesene and an other unknown sesquiterpenoid. The relation between these three compounds was demonstrated previously in cucumber (Kappers et al., 2010), and it suggests that they are products of a single terpene synthase.

Although individual spider mite-induced plant volatiles have no *a priori* function, and predatory mites can learn to associate odors with the presence of prey after a brief learning experience of less than 15 min (Van Wijk et al., 2008), our study demonstrates that the composition of the induced blend matters to the plant. Within a large spatial greenhouse setup with a high three-dimensional complexity, as in this study, predatory mites preferred certain accessions above others. An accession that emits an attractive induced blend at low rates of herbivory during initial infestation, will attract more bodyguards that can quickly build up a population at the location where the herbivore is settling. It is, therefore, worthwhile to select and breed for cucumber varieties that have a strong attraction of natural enemies. This study identified some of the volatiles that affect natural enemy behavior and that could be used as markers in breeding. Furthermore, we demonstrated that highly correlated compounds in the emitted volatile blend may have opposite effects on attractiveness. The introduction of varieties that improve the foraging success of natural enemies could be one of the critical factors that can further enhance the success rate of biological control.

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Pheromone Communication in *Nasonia vitripennis*: Abdominal Sex Attractant Mediates Site Fidelity of Releasing Males

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Abstract Males of the parasitic wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae) use a substrate-borne sex pheromone to attract virgin females. The pheromone is synthesized in the rectal vesicle and deposited via the anus by dabbing movements of the abdominal tip. The chemicals attracting the females are composed of a mixture (4*R*,5*R*- and (4*R*,5*S*)-5-hydroxy-4-decanolides (HDL) being synergized by the trace component 4-methylquinazoline (4-MeQ) which is not attractive for females when offered alone. Here we show that male pheromone deposits are not only attractive to virgin females but also for the releasing males themselves. In an olfactometer bioassay, males were strongly attracted by their own pheromone markings but were unable to discriminate between their own markings and those deposited by other males. Polar fractions of pheromone gland extracts containing the HDLs and 4-MeQ were also highly attractive for males. Bioassays using synthetic pheromones in natural doses revealed that combinations of HDL/4-MeQ and 4-MeQ alone attracted males whereas the HDLs alone were behaviorally inactive. Furthermore, males did not discriminate between HDL/4-MeQ and 4-MeQ alone. We conclude that the trace component 4-MeQ mediates site fidelity of *N. vitripennis* males at sites previously marked with the abdominal sex pheromone. The use of 4-MeQ to stay at and to return to scent-marked patches rather than marking new ones might be a strategy to economize semiochemical use in *N. vitripennis* males.

Key Words Sex pheromone · Abdomen dipping · Site fidelity · Mate finding · Pteromalidae · Parasitic wasp

Introduction

The use of pheromones for sexual communication is often associated with costs for the releasing gender. These comprise physiological costs if the attractive chemicals are biosynthesized from limited resources and ecological costs if the signal not only attracts potential mates but also natural enemies and intraspecific competitors (Wyatt, 2003; Wertheim et al., 2005). Hence, sex pheromone release has to be economical in space and time, i.e. restricted to sites and situations in which receptive mates are likely to occur. For species depositing the attractive chemicals on substrates rather than releasing them directly from the producing gland, site fidelity is of crucial importance for successful mate attraction (Raw, 1975; Kroiss et al., 2010). If scent-marked areas attract also opportunistic conspecifics of the same sex, site-fidelity might pass into territoriality, i.e. scent-marked holdings are defended aggressively against the intruders (Baker, 1983). The mechanisms involved in chemically mediated site fidelity and territoriality in insects are often not fully understood, although it is self-evident that individuals may use the same chemicals they deposited for mate attraction to recognize and defend their holdings.

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The Jewel wasp *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) is a gregarious ectoparasitoid parasitizing pupae of numerous cyclorrhaphous flies (Whiting, 1967). The species is found worldwide in nests of hole-breeding birds. Males of *N. vitripennis* are protandrous and remain on or near the host they emerged from to mate with the females emerging shortly after. Females of *N. vitripennis* typically mate only once, and males compete for them aggressively. Dominant males defend the escape hole against their competitors (van den Assem et al., 1980a; van den Assem, 1986; Leonard and Boake, 2006). Subordinate other males (so-called satellite males) often wait in the vicinity of the host puparium for females leaving the natal site without being inseminated by the dominant male (van den Assem, 1986). Attraction and arrestment of virgin females is achieved by an abdominal sex attractant produced in the abdomen of males. It consists of a mixture of (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolides (HDL) (Ruther et al., 2007) being synergized by the trace component 4-methyquinazoline (4-MeQ). In contrast to the HDLs, 4-MeQ is not attractive to females when offered alone (Ruther et al., 2008). The pheromone is synthesized in the rectal vesicle (Abdel-latief et al., 2008) and deposited on the ground by dabbing movements ("abdomen dipping") of the abdominal tip (see supplementary online material) (Barrass, 1969; van den Assem et al., 1980b; Steiner and Ruther, 2009). Pheromone deposits remain attractive for about 2 hrs and the range of activity is about 5 cm (Steiner and Ruther, 2009). Marking behavior is shown increasingly by males after mating or mere contact with a receptive female (van den Assem et al., 1980b; Steiner and Ruther, 2009). This suggests that males economize the use of the pheromone by releasing it specifically if the target is present. Van den Assem et al. (1980b) observed that males were arrested for days on areas scent-marked by themselves suggesting that they use the substrate borne sex pheromone not only to attract virgin females but also to recognize their territorial holdings. In the present paper we investigate the chemical basis of the site fidelity in *N. vitripennis* males. We identify the responsible chemical and study whether males are able to discriminate their own markings from those deposited by other males. We discuss the results with respect to the biology and the mating system of *N. vitripennis.*

Methods and Materials

Insects and General Methods Parasitoids used in this study originated from the same *N. vitripennis* strain used in our previous pheromone studies (Ruther et al., 2007, 2008, 2009; Steiner and Ruther, 2009). Starter individuals were collected from a bird's nest in Hamburg, Germany. Parasitoids were reared on pupae of the green bottle fly *Lucilia caesar* (Diptera: Calliphoridae) at 25°C, 60% RH, and a photoperiod of 16:8 (L/D) (for details see Ruther et al., 2007). Virgin parasitoids of either sex were obtained by excising pupae from host puparia 1–2 d prior to eclosion and keeping them in 1.5 ml microcentrifuge tubes (Brand GmbH, Wertheim, Germany). Male wasps used in the bioassays were 2 d old and were generally of equal size (660–700 μm) to control for size-dependent differences in the pheromone titers (Ruther et al., 2007). Size was determined by measuring the head width of fully melanized pupae. One hr prior to the experiments, parasitoids were held at room temperature (20–22°C) for acclimatization. Handling of the parasitoids was done using a fine artist's paint brush. Each parasitoid was used only once.

Bioassays The response of males to naturally deposited pheromone markings, fractionated pheromone gland extracts and synthetic pheromone components was investigated by using a still air 4-cavity olfactometer made from acrylic glass (Steiner and Ruther, 2009). It consisted of a round walking arena (9 cm diameter, 8 mm thick) with four symmetrically arranged spherical cavities (1 cm diameter, 4 mm deep), a low rim (2 mm high) and a glass lid to cover the arena. Only two opposite cavities of the olfactometer were used to offer the stimuli to be compared. After application of the stimuli, males were introduced singly into the centre of the olfactometer which then was covered by the glass lid. Subsequently, the residence time of the test males in the cavities to be compared was recorded for 5 min using The Observer 3.0 observational software (Noldus, Wageningen, The Netherlands) (N=20 for all bioassays). After each experiment, the male was removed and the olfactometer was cleaned with ethanol and demineralized water. To avoid bias due to optical stimuli, the olfactometer was placed into a white plastic bowl (30 cm length x 30 cm width x 15 cm height) and illuminated from above with a 60 W bulb. Additionally, the olfactometer was turned clockwise by 90° after every run.

In the first experiment, we tested the response of males to their own naturally deposited pheromone markings. Previous studies have demonstrated that males show a clearly increased pheromone marking activity after mating (Steiner and Ruther, 2009). Thus males were allowed to mate with a virgin female prior to the experiment. For this purpose a couple was introduced into the cavity which was covered by a microscope cover slip. After observation of the copulation, the female was removed and the male was allowed to mark the cavity for 5 min. After marking, males were removed from the olfactometer and kept isolated for 3 min in a clean glass dish. After this time, the male was re-transferred to the olfactometer and the behavior was observed as described above. The second experiment was performed following the same protocol but the pheromone

deposits offered originated from a foreign male. Then, we exposed males simultaneously to their own pheromone deposits and to those from a foreign male. Both markings were deposited at the same time. Furthermore, we tested whether the arrestment of males is in fact mediated by components of the known pheromone gland (rectal vesicle). For this purpose, we offered to males in one cavity one individual equivalent of a fractionated pheromone gland extract (methanol fraction applied to filter paper, see below) and the pure solvent in the other.

Finally, we tested the response of males to synthetic analogues of the known pheromone compounds (4*R*,5*R*)-HDL, (4*R*,5*S*)-HDL, and 4-MeQ, respectively. We tested a combination of 100 ng HDL [1:1 mixture of (4*R*,5*R*)-HDL and (4*R*,5*S*)-HDL] plus 1 ng 4-MeQ. The pheromone dose represents about one third of a male abdomen equivalent and the ratio of approximately 100:1 for HDL and 4-MeQ was found in the male abdomen in a previous study (Ruther et al., 2008). We also tested equal doses of HDL and 4-MeQ alone and investigated whether the combination of 100 ng HDL and 1 ng 4-MeQ is more attractive than 4-MeQ alone. (4*R*,5*R*)-HDL was synthesized as described by Garbe and Tressl (2003) by Sharpless asymmetric dihydroxylation of ethyl (4*E*)-decenoate. After oxidation of the hydroxyl group using Dess-Martin periodinane, the resulting (4*R*)-5-oxo-4-decanolide was reduced by sodium borohydride to obtain a 1:1 mixture of (4*R*,5*R*)-HDL and (4*R*,5*S*)-HDL (Garbe and Tressl, 2003). 4-MeQ was synthesized following the protocol of Bergmann et al. (1986) by reaction of 2-aminobenzonitrile with methyl magnesium iodide and subsequent treatment with ethyl formate.

Fractionation of Pheromone Gland Extract The rectal vesicles of 30 freeze-killed males were dissected under a stereo microscope as described elsewhere (Abdel-latief et al., 2008). The glands were transferred to an autosampler vial with micro insert and extracted for 30 min with 100 µl dichloromethane. The extract was carefully concentrated under nitrogen to a volume of 10 µl and diluted with 70 µl hexane. The extract was applied to a conditioned 100 mg silica gel cartridge (IST, Mid-Glamorgan, UK) and eluted with 500 µl each of hexane, dichloromethane, and methanol. The methanol fraction containing the pheromone components was carefully dried under a gentle stream of nitrogen and the residue was re-solved with 30 µl of dichloromethane. This extract was used for bioassay as described above. The presence of the *N. vitripennis* sex pheromone components was verified by GC-MS using the instrumentation and conditions described elsewhere (Ruther et al., 2007).

Statistical Analyses Statistical analyses were performed using Statistica 4.5 scientific software (StatSoft, Tulsa, OK,

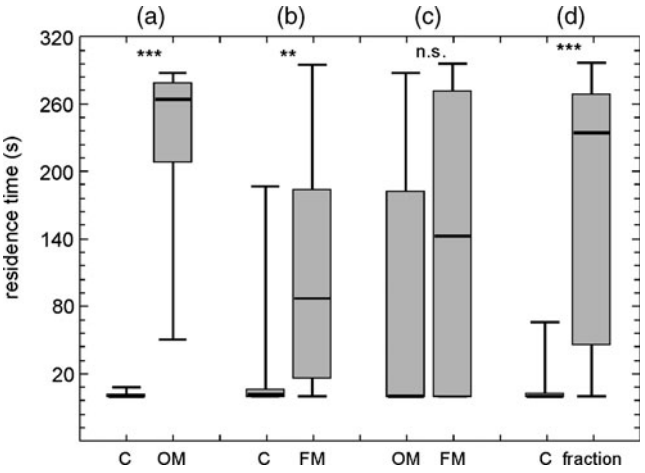


Fig. 1 a–d. Residence times of *N. vitripennis* males in a still air olfactometer during a 5 min observation period. Prior to the experiment, the test cavities were marked for 5 min with the abdominal sex attractant by the test male itself (own marking, OM) or by another male (foreign marking, FM) (**a**–**c**) or were treated with 1 individual equivalent of a fractionated pheromone gland extract (**d**). Control cavities (C) remained untreated (**a**–**c**) or were treated with pure solvent (**d**), respectively. Box and whisker plots indicate the median (horizontal line) with 25th and 75th percentiles (*boxes*) and maximal/minimal values (whiskers). Asterisks indicate significant differences between the test and the control cavities at $P<0.01$ (**) or $P<0.001$ (***) (one-sample Wilcoxon test, n.s. = not significant, $N=20$)

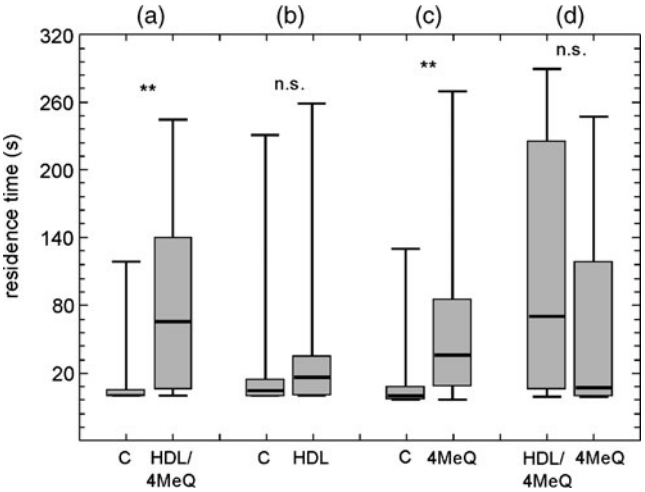


Fig. 2 a–d. Residence times of *N. vitripennis* males in a still-air olfactometer during a 5 min observation period. Prior to the experiment, the cavities were equipped with disks of filter paper treated with the synthetic sex pheromone components HDL and/or 4-MeQ, respectively. Control cavities (C) were treated with pure solvent. Box and whisker plots indicate the median (*horizontal line*) with 25th and 75th percentiles (*boxes*) and maximal/minimal values (*whiskers*). Asterisks indicate significant differences between test and control cavities at $P<0.01$ (**) (one-sample Wilcoxon test, n.s. = not significant, $N=20$)

U.S.A). Residence time of males spent in the cavities to be compared was analyzed by a one-sample Wilcoxon test.

Results

Males of *N. vitripennis* spent significantly more time in the pheromone-marked cavity of the olfactometer than in the control cavities irrespective of whether the pheromone deposits originated from themselves (Z=3.92, P<0.001, Fig. 1a) or from foreign males (Z=2.688, P<0.01, Fig. 1b). When offered simultaneously, males were not able to discriminate their own pheromone markings from foreign ones (Z=1.248, P=0.212, Fig. 1c). Polar fractions of the male sex pheromone glands containing the HDLs and 4-MeQ were also attractive to males (Z= 4.124, P< 0.001, Fig. 1d). Males spent significantly more time in cavities treated with a synthetic mixture of (4*R*,5*R*)-HDL, (4*R*,5*S*)-HDL and 4-MeQ (Z=2.931, P=0.0034, Fig. 2a) and with 4-MeQ alone (Z=3.034, P=0.0024, Fig. 2c) whereas the HDLs were behaviorally inactive, when tested alone (F= 0.977, P=0.329, Fig. 2b). When offered simultaneously, the combination of HDLs/4-MeQ was not more attractive to males than 4-MeQ alone (Z=1.471, P=0.141, Fig. 2d).

Discussion

The present study demonstrates that site fidelity of *N. vitripennis* males at scent-marked areas is mediated by 4-MeQ, the minor component of their abdominal sex pheromone. In contrast, the HDLs which have been shown to be the primary attractants of virgin females did not contribute to the response of males to their own pheromone deposits. Sex pheromones should be honest signals and thus, the biosynthesis typically causes physiological costs (Johansson and Jones, 2007). In the case of the HDLs, these costs may arise from the pheromone precursor linoleic acid (J. R., unpublished data) which is an essential nutrient and a limited resource for most animals (Wathes et al., 2007, but see Borgeson et al., 1991; Weinert et al., 1993). Male pheromone markings remain attractive for virgin females for at least 2 hrs (Steiner and Ruther, 2009). Hence, using 4-MeQ to stay at scent-marked patches rather than dispersing and marking new ones might be a strategy of *N. vitripennis* males to save costly resources. 4-MeQ allows them to stay where attracted females arrive and helps them to return to the scent-marked areas which they often leave when chasing females over some distance during courtship or when chasing male competitors away from their territorial holdings (van den Assem et al., 1980a, b). Pheromone mediated site fidelity might be part of the mating strategy of both the dominant male and the subordinate satellite males. The dominant male may minimize the number of females dispersing unfertilized and the satellite males may compete in a kind of chemical contest for those females that nevertheless leave the natal site without immediate attention of the dominant male (van den Assem et al. 1980a). The chemical contest scenario is supported by the fact that virgin females orientate along concentration gradients of the male sex pheromone and are able to discriminate even subtle concentration differences (Ruther et al., 2009). Hence, males being able to deposit the sex attractant in higher doses than their competitors might get their mating opportunities without getting involved in aggressive interactions with dominant males at the eclosion holes of host puparia. However, the fact that males do not discriminate between their own pheromone deposits and those from other males, suggests that, apart from the physiological costs of pheromone biosynthesis, males have to face also ecological costs by attracting opportunistic males. These might compete for the attracted females without releasing pheromone themselves. Detailed behavioral observations under controlled near-natural conditions are necessary to unravel the exact role of scent-mark mediated site fidelity in the mating strategies of dominant and subordinate *N. vitripennis* males.

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The Absolute Configuration of the Sex Pheromone of the Citrophilous Mealybug, *Pseudococcus calceolariae*

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Abstract The absolute configuration of the sex pheromone of the citrophilous mealybug, *Pseudococcus calceolariae*, was determined to be (*1R,3R*)-[2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropyl]methyl (*R*)-2-acetoxy-3-methylbutanoate. NMR, derivatization reactions, chiral gas chromatography-mass spectrometry, and comparison with synthetic chiral reference compounds, were used to determine the absolute configuration of this compound. This activity of this compound was further confirmed by testing synthetic stereoisomers of the compound as lures in traps for adult male mealybugs. Traps baited with 1,000 μg of the pheromone compound caught 36 times more males than traps baited with virgin females. A mixture of stereoisomers of the pheromone compound can be used for field trapping without adverse effects on trap catch. A comparison with the structures of other sex pheromones of mealybugs is presented.

Key Words (*R,R,R*)-Chrysanthemyl 2-acetoxy-3-methylbutanoate · (*R,R,R*)-chrysanthemyl 2-hydroxy-3-methylbutanoate · Chrysanthemol · Mealybug pheromone · *Pseudococcus calceolariae*

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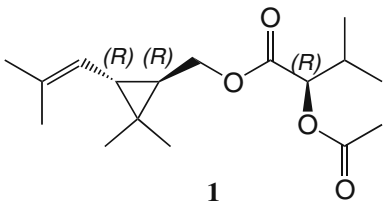
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Introduction

The citrophilous mealybug, *Pseudococcus calceolariae* (Maskell, Hemiptera: Pseudococcidae), is a pest of numerous economically important crops, such as orange, lemon, avocado, and other vegetable, fruit and floricultural plants (Bartlett, 1978). The insect is a problem for agricultural crops in a number of ways, including vectoring of viruses, such as the *Grapevine Leafroll-associated Virus 3* (GLRaV-3), through causing direct damage to plants by its piercing-sucking feeding habit, which can lead to malformation of fruits, shoots, or leaves, and transmission of other diseases, and through the excretion of honeydew, which facilitates the development of fungi affecting fruit quality. Furthermore, the insect is responsible for additional economic loss to Chilean agriculture through the quarantine restrictions imposed by a number of fruit-importing countries.

The sex pheromone of *P. calceolariae* recently was identified as chrysanthemyl 2-acetoxy-3-methylbutanoate (**1**) (El-Sayed et al., 2010). This paper reports the determination of the absolute configuration of the insect-produced compound, and the results of field trials testing the attractiveness of stereoisomers of the pheromone to adult males.



1

Methods and Materials

Insects Adult and immature stages of *P. calceolariae* were collected from vineyards in Hawke's Bay, New Zealand, and from raspberry plantations near Quillota, Chile, and reared on sprouted seed potatoes or butternut squash. Virgin females were obtained as described earlier (El-Sayed et al., 2010).

Volatile Collection Mature virgin females were transferred into glass air-entrainment chambers, and headspace chemicals trapped on Tenax (50 mg) or activated charcoal (150 mg). Glass chambers, containing clean rearing substrate or rearing substrate infested with mixed-sex colonies, were used as controls. The trapped volatiles were eluted with 1 ml hexane every 4–7 day, and the extracts stored at −20°C until further use.

Chemical Analyses ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AMX 400 instrument. Chemical shifts are expressed in ppm relative to tetramethylsilane. Analyses of female headspace volatiles and synthetic chemicals were carried out by coupled gas chromatography/mass spectrometry, (GC/MS), generally with a Saturn 2200 instrument (Varian Walnut Creek, CA, USA) in electron impact mode at 70 eV. The following capillary columns were used for the analyses: 1) a 30 m×0.25 mm i.d.×0.25 μm film thickness VF-5 ms, non-polar column (Varian); 2) a 30 m×0.25 mm i.d.×0.25 μm VF-23 ms, polar column (Varian); 3) a 30 m×0.25 mm i.d.×0.25 μm CycloSil-β chiral column (J&W Scientific); 4) a 30 m×0.25 mm i.d.×0.25 μm RT-γ-DEXsa column (Restek). The injectors were held at 220°C and the injections were splitless for 0.6 min. The oven was programmed from 40°C (held for 2 min) to 220°C at 4°C.min^{-1}. Helium was used as carrier gas at a flow rate of 1 ml.min^{-1}. A Shimadzu GC-17A/GCMS-QP5050A combination, equipped with a 30 m x 0.25 mm HP-5 fused silica capillary column, was used for some analyses.

Chemical Syntheses Reagents and solvents were purchased from Sigma-Aldrich (St Louis, MO, USA) and Merck (Gibbtown, NJ, USA) and used without further purification. Column chromatography was carried out on silica gel 60 (0.040–0.063 mm, Merck) using mixtures of hexane and ethyl acetate as eluents.

(1R,3R)-Chrysanthemol (1*R*,3*R*)-Chrysanthemol was obtained by reducing commercially available (1*R*,3*R*)-chrysanthemic acid with $LiAlH_4$ in dry Et_2O at room temperature under standard conditions.

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.89 (1 H, m), 3.80 (1 H, dd *J* =11.4, 6.5 Hz), 3.59 (1 H, dd *J* =11.4, 8.5 Hz), 1.72 (3 H, s), 1.69 (3 H, d *J* =1.1 Hz), 1.17 (3 H, s), 1.14 (1 H, dd *J* =8.0, 5.2 Hz), 1.08 (3 H, s), 0.86 (1 H, ddd *J* =8.5, 6.5, 5.3 Hz) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 133.16, 123.38, 63.72, 35.19, 28.68, 25.61, 22.68, 22.33, 21.37, 18.26 ppm.

(1R,3R)-Chrysanthemyl (R)-2-acetoxy-3-methylbutanoate (1*R*,3*R*)-Chrysanthemyl (*R*)-2-acetoxy-3-methylbutanoate was prepared following the procedure previously described for the isomeric mixture (El-Sayed et al., 2010). (*R*)-2-Hydroxy-3-methylbutanoic acid was acetylated using acetic anhydride in pyridine and a catalytic amount of DMAP. The resulting product was transformed to the acid chloride [$(COCl)_2$, DMF, benzene], which was esterified with (1*R*,3*R*)-chrysanthemol, giving the target compound in 49% overall yield. Chemical purity: 93% (GC).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.86 (1 H, m), 4.84 (1 H, d J=4.6 Hz), 4.48 (1 H, dd J=11.7, 6.7 Hz), 3.97 (1 H, dd J=11.7, 9.0 Hz), 2.24 (1 H, dhept J=6.9, 4.6 Hz), 2.15 (3 H, s), 1.71 (3 H, s), 1.67 (3 H, m), 1.19 (1 H, dd J=7.8, 5.3 Hz), 1.15 (3 H, s), 1.05 (3 H, s), 1.02 (3 H, d J=6.9 Hz), 1.00 (3 H, d J=6.9 Hz), 0.90 (1 H, ddd J=9.0, 6.7, 5.3 Hz) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.69, 169.81, 133.69, 122.74, 76.86, 66.36, 30.88, 30.05, 29.19, 25.56, 22.46, 22.29 21.54, 20.59, 18.63, 18.26, 17.26 ppm.

Ethyl (R)- and (S)-2-hydroxy-3-methylbutanoate Commercial (*R*)- or (*S*)-2-hydroxy-3-methyl-butanoic acid (8 mg, 0.068 mmol) was dissolved in ethanol (2.5 ml), and a drop of conc. H_2SO_4 added. After stirring overnight, the reaction mixture was partitioned between a water layer and a hexane layer. The hexane layer was dried ($MgSO_4$), filtered, concentrated, and analyzed by GC/MS.

MS (70 eV): *m/z* (rel. intensity)=(*R*-isomer): 147 (32), 119 (12), 104 (18), 76 (38), 73 (100), 58 (14), 55 (56), 41 (12), and 39 (20). (*S*-isomer): 147 (33), 119 (12), 104 (18),

76 (39), 73 (100), 58 (16), 55 (55), 54 (18), 41 (12), and 39 (23).

Transesterification of the Mealybug Pheromone Extract A sample containing 4,000 female day equivalents of mealybug pheromone extract was concentrated under a stream of argon to near dryness. One milliliter of calcium ethoxide solution [prepared from 10% CaH_2/ethanol (w/v)] was added, and the mixture left to react overnight. Dichloromethane and water were added, and the organic layer dried ($MgSO_4$), filtered, and submitted to analysis.

Field Trapping Two experiments were carried out to test the activity of various isomers of chrysanthemyl 2-acetoxy-3-methylbutanoate for the attraction of *P. calceolariae*.

Experiment 1: Treatments were: 10 μg of a mixture of the four stereoisomers of chrysanthemyl (*R*)-2-acetoxy-3-methylbutanoate, 10 μg of a mixture of the four stereoisomers of chrysanthemyl (*S*)-2-acetoxy-3-methylbutanoate, and 10 μg of an 8-component mixture of the stereoisomers. Five virgin females were used as positive controls, and a blank trap was used as a negative control. The experiment was carried out in February 2009 (New Zealand) and April 2009 (Chile).

Experiment 2: Treatments were five doses of pure (1*R*,3*R*)-chrysanthemyl (*R*)-2-acetoxy-3-methylbutanoate (0, 0.1, 1, 10, 100, and 1,000 μg), and a 1,000 μg dose of the 8-stereoisomers mixture. The amount of the *RRR*-isomer in this mixture was ≈210 μg. Traps baited with 5 virgin females were used as positive controls. Experiments were carried out in March 2009 (New Zealand) and late April/early May 2009 (Chile).

The experiments were carried out in vineyards in Hawke's Bay, New Zealand (39°65′S 176°83′E) and in a raspberry plantation near Quillota, Chile (32°43′S 71°11′ W). For preparation of lures, rubber septa were treated with the compounds dissolved in 200 μl hexane, with the solvent allowed to evaporate in a fume hood. Septa were stored at −20°C until use. Septa were placed in the center of the white sticky base of the trap. Adopting a randomized block design, traps were placed in five rows, with five replicates per treatment, with 15-m spacing between trapping stations and treatment rows. Each of the treatments tested was randomly assigned to a trap in a row of vines. The effect of treatment on the mean number of males captured was tested using ANOVA (SAS Institute Inc. 1998) after variances were stabilized using the $\sqrt{x+1}$ transformation. Significantly different treatment means were identified using Fisher's Protected Least Significant Difference Test (Statview, SAS Institute Inc., Cary, NC, USA).

Results

Chemical Analyses Headspace volatiles from mature virgin females contained three compounds that were not found in control samples. These compounds have been previously identified as [2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropyl]methanol (chrysanthemol), chrysanthemyl 2-hydroxy-3-methyl-butanoate, and chrysanthemyl 2-acetoxy-3-methylbutanoate (**1**) (El-Sayed et al., 2010).

Mealybug-produced chrysanthemyl 2-acetoxy-3-methylbutanoate showed only one GC peak on each of the non-polar, polar, and two chiral columns (β- and γ-cyclodextrin). In order to determine the relative configuration of the alcohol part of the compound, a commercial sample of chrysanthemol, containing a mixture of *cis* and *trans* isomers, was analyzed by NMR and GC/MS; this revealed that the mixture had a 1:2 ratio of diastereomers. The proportion of the signals of the C-1 ring proton at 0.83 (*trans*) and 1.38 (*cis*) ppm, and of the olefinic proton at 4.86 (*trans*) and 4.96 (*cis*), in the NMR spectrum showed that the smaller peak on the GC/MS trace corresponded to the *cis* isomer (Bramwell et al., 1969; Takano et al., 1985). Analysis of a hydrolyzed (KOH, EtOH) mealybug headspace extract on non-polar and polar columns showed that the insect-produced

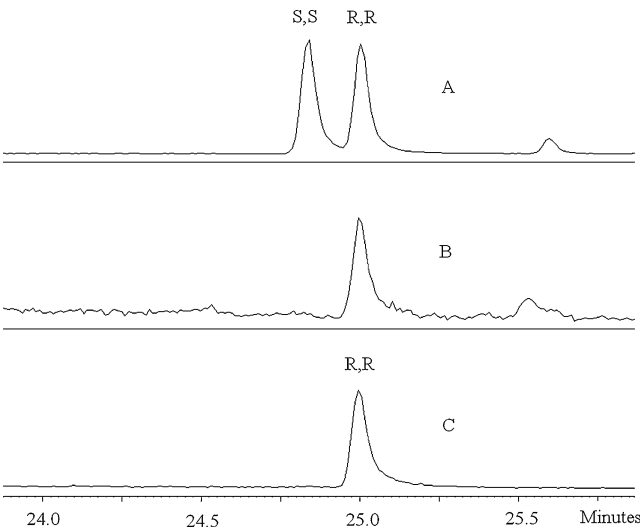
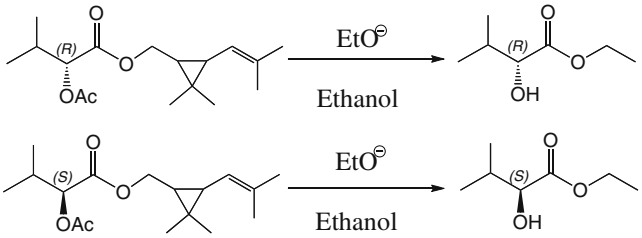


Fig. 1 Gas chromatograms of **a** synthetic racemic chrysanthemol (90% *trans* and 10% *cis*), **b**, natural chrysanthemol from the hydrolyzed extracts of headspace volatiles collected from sexually mature female citrophilous mealybugs, and **c**, synthetic *R,R*-chrysanthemol



Scheme 1 Solvolysis of the mealybug pheromone stereoisomers by ethoxide/ethanol

chrysanthemol matched the retention time of the *trans* isomer, showing that the compound possessed either the (1*R,*3*R*) or the (1*S,*3*S*) configuration on the alcohol part of the ester.

To determine the absolute configuration of the alcohol part, an isomeric mixture of chrysanthemol [racemates of *trans* (90%) and *cis* (10%)] was analyzed by GC/MS on the β-cyclodextrin column; both *trans* enantiomers separated from each other (Fig. 1a). The chrysanthemol isomer found in the hydrolyzed mealybug extract eluted at the same retention time as the second peak of *trans*-chrysanthemol (Fig. 1b). Synthetic (1*R,*3*R*)-chrysanthemol also matched the retention time of the second peak (Fig. 1c), proving that the absolute configuration of the alcohol part of the pheromone was *RR*. Additionally, the retention time of (1*R*,3*R*)-chrysanthemol matched that of the mealybug-produced compound on a second chiral column (γ-cyclodextrin, data not shown).

To determine the configuration of the acid part of the pheromone compound, commercially available (*R*)- and (*S*)-2-hydroxy-3-methylbutanoic acids were converted to their corresponding ethyl esters. These esters separated well

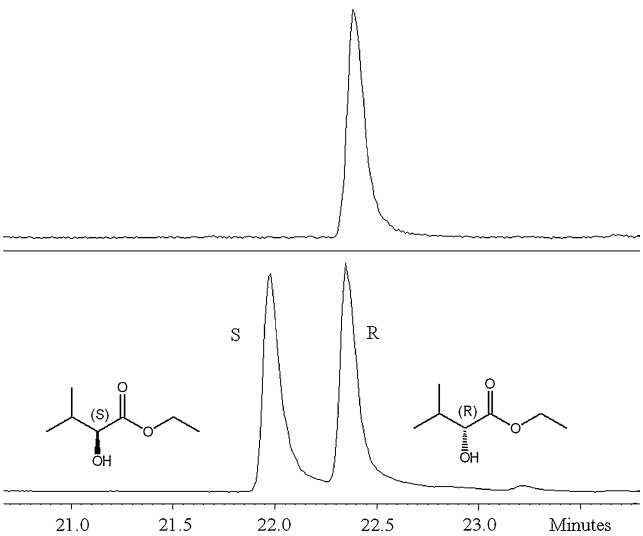


Fig. 2 GCMS trace of ethyl (*R*)-2-hydroxy-3-methylbutanoate produced by solvolysis of the mealy bug pheromone using SIM-mode *m/z* 73 (*top*); GCMS trace of racemic ethyl 2-hydroxy-3-methylbutanoate (*below*)

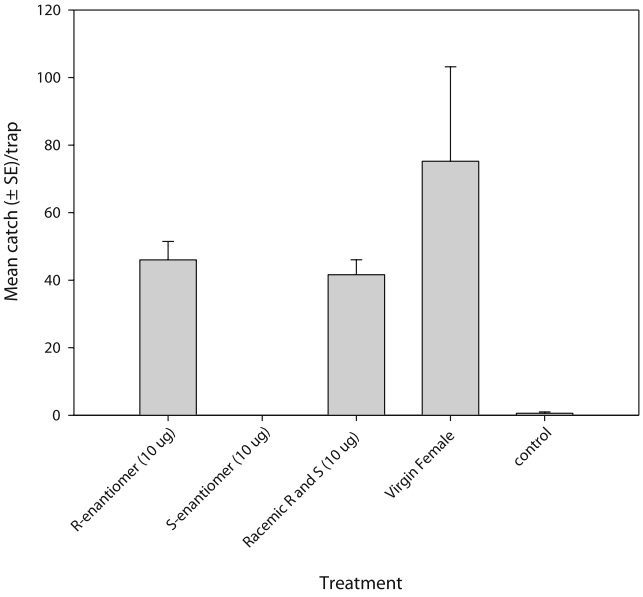


Fig. 3 Mean catch (± SE) of male citrophilous mealybugs in red delta traps baited with the following chemicals: 10 μg of a mixture of the four esters synthesized from *R*-acetate and *cis/trans* chrysanthemol (1:2 mixture of geometric isomers), 10 μg of a mixture of the four esters synthesized from the *S*-acetate and *cis/trans* chrysanthemol (1:2 mixture of geometric isomers), a mixture of all eight esters (from R- and S-acetates), virgin females, and a blank (control) trap. Different letters atop columns indicate significant differences (FPLS Test, $\alpha = 0.05$)

on the chiral columns. Subsequently, the mealybug headspace extract was subjected to a transesterification reaction (calcium ethoxide in ethanol) to convert the insect-produced chrysanthemyl 2-acetoxy-3-methylbutanoate to ethyl 2-hydroxy-3-methylbutanoate (Scheme 1). Gas chro-

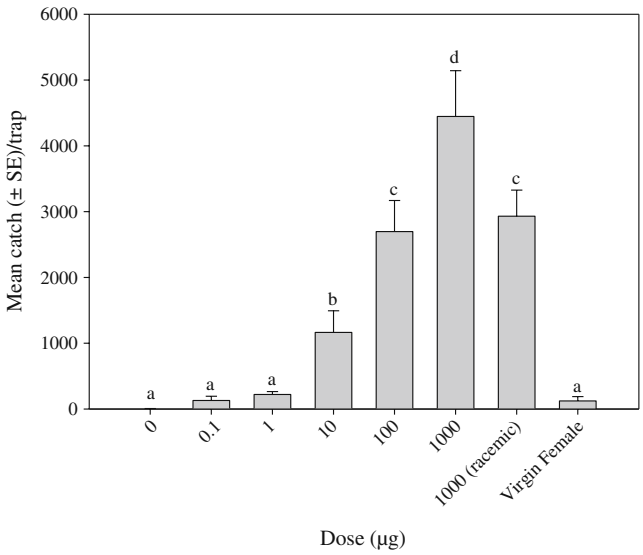


Fig. 4 Mean catch (± SE) of adult male citrophilous mealybugs in red delta traps baited with various dosages of (1*R*,3*R*)-chrysanthemyl (*R*)-2-acetoxy-3-methylbutanoate loaded on red rubber septa. Different letters atop columns indicate significant differences (FPLS Test, $\alpha = 0.05$)

matographic analysis on the chiral columns showed a single isomer that co-eluted with ethyl (*R*)-2-hydroxy-3-methylbutanoate (Fig. 2).

Field Trapping In the first experiment, 10 μg of the 4-component mixture, that contained the stereoisomers with *R* configuration in the acid part of the molecule (amount of *RRR* stereoisomer, ca. 3 μg), or of a mixture of all eight stereoisomers (amount of *RRR* stereoisomer, ca. 1.5 μg) both attracted males (relative to the control). The mean numbers of males captured by these traps did not differ from that caught in traps baited with virgin females. The stereoisomeric 4-component mixture with *S* configuration in the acid part of the ester did not attract males (Fig. 3).

In the second experiment, traps baited with doses of 10 μg or greater of the pure *RRR* stereoisomer, caught more males than the control or virgin female traps. In general, the number of males caught in traps increased with increasing dose (up to 1,000 μg) of the pure *RRR* stereoisomer. A 1,000 μg dose of the mixture of the 8 stereoisomers (containing ca. 210 μg *RRR* isomer) attracted a number of males similar to the 100 μg dose of the pure *RRR* isomer (Fig. 4).

Discussion

Chemical analysis and field tests showed conclusively that virgin females of the citrophilous mealybug produce (1*R*,3*R*)-chrysanthemyl (*R*)-2-acetoxy-3-methylbutanoate, and that this compound is highly attractive to conspecific males. Further, field experiments demonstrated that traps baited with relatively low doses of the *RRR* isomer caught as many males as traps baited with virgin females. This is in

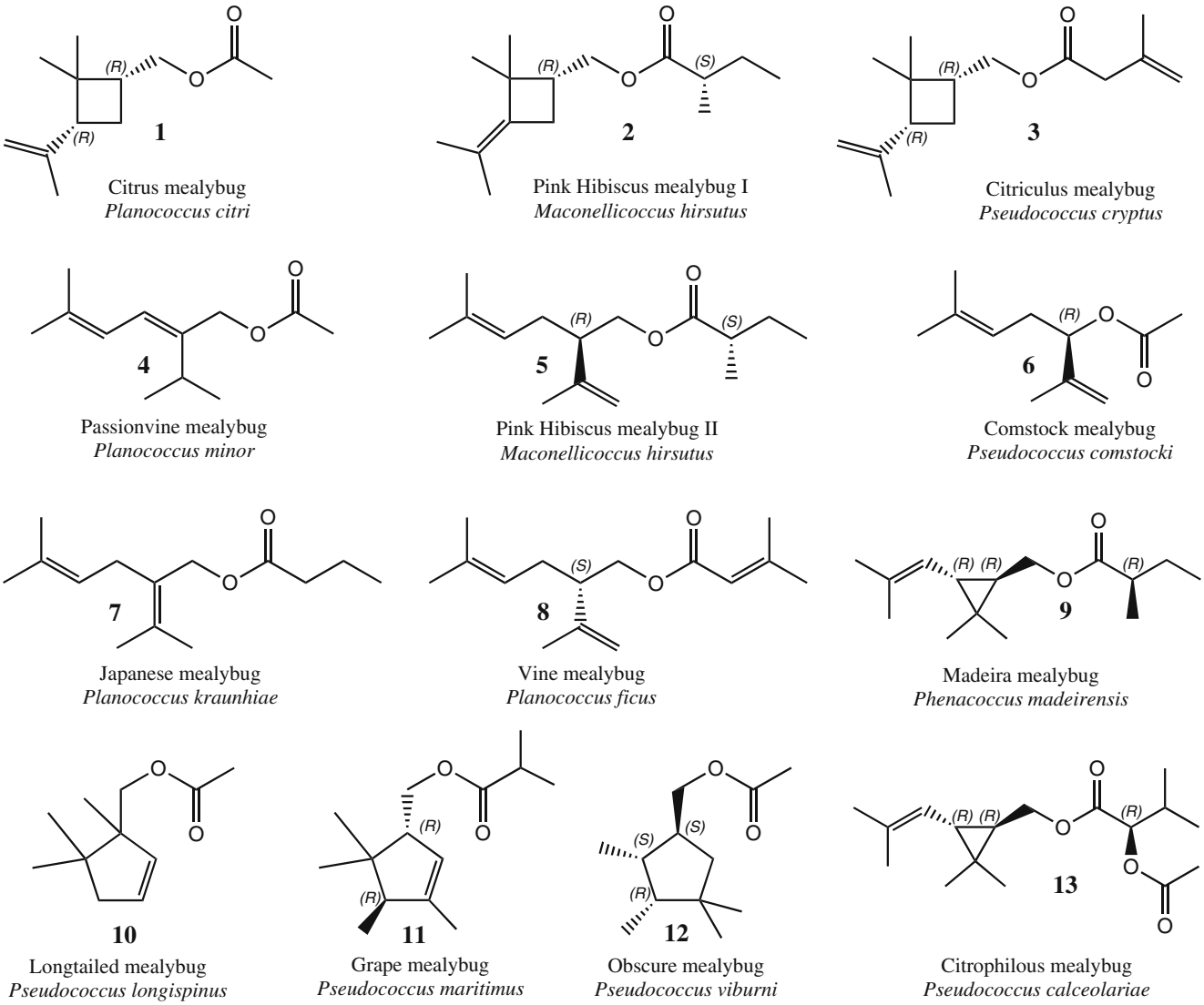


Fig. 5 The known mealybug pheromone compounds (El-Sayed, 2009; Millar et al., 2005a)

accord with the high sensitivity of male mealybugs to female pheromone reported for other species (e.g., Zhang and Amalin, 2005). This compound is the second example of a chrysanthemol derivative produced by an insect: a component of the sex pheromone of the Madeira mealybug, *Phenacoccus madeirensis*, is (1*R*,3*R*)-chrysanthemyl (*R*)-2-methylbutanoate (Ho et al., 2009). It is noteworthy that the chrysanthemol part has the same absolute configuration as in the *P. calceolariae* compound, but is linked to a different chiral acid.

When we tested various mixtures of stereoisomers in field trials, results showed that the *R* configuration in the acid part of the ester was necessary for activity, and that stereoisomers with the *S* configuration in the acid were not attractive on their own, or were inhibitory in a mixture with the active stereoisomer. Additionally, traps baited with 1,000 μg of a mixture of all 8 stereoisomers (containing ca. 210 μg of the *RRR* isomer) captured a number of males similar to traps baited with 100 μg of pure *RRR* isomer, also suggesting that the non-natural stereoisomers are not, or at most only slightly, inhibitory. That isomeric mixtures of the pheromone can be used for monitoring the citrophilous mealybug is important, as the relatively inexpensive racemic chrysanthemol can be used for the synthesis of the pheromone rather than the enantiomerically pure and expensive precursor (1*R*,3*R*)-chrysanthemic acid.

In general, mealybugs do not seem to use mixtures of stereoisomers in their pheromones, either for behavioral synergism or antagonism (against sibling species). Instead, mealybug pheromones appear to differ from sibling species by structural isomerism. Sex pheromones now have been identified for twelve mealybug species, including the citrophilous mealybug pheromone. The core structures for all are irregular, non-head-to-tail, monoterpenols, esterified to a C2-C5 acid (Fig. 5). Five of the hitherto-known mealybug pheromones consist of compounds with a C5 isoprenic acid part, four are acetates, and two are butanoates. Four of the pheromone compounds (2,5,9,13; Fig. 5), have a stereogenic center in the acid part. The alcohol part of the pheromone compounds is a monoterpenol (or, as in the case of *P. comstocki*, a norterpenol), and it is related to maconelliol (compounds 1–3, Fig. 5; Bierl-Leonhardt et al., 1981; Arai et al., 2003; Nakahata et al., 2003; Zhang et al., 2004a, b, 2006; Zhang and Amalin, 2005), lavandulol (compounds 4-8; Negishi et al., 1980; Hinkens et al., 2001; Zhang et al., 2004a, b, 2006; Zhang and Amalin, 2005; Ho et al., 2007; Sugie et al., 2008), cyclopentylmethanol (compounds 10–12; Millar et al., 2005b, 2009; Figadere et al., 2007), or chrysanthemol (9,13; Ho et al., 2009 and the present paper).

Future work will investigate the role of structural- and stereo- isomerism in the specificity of the pheromone signals of mealybugs.

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cis-Vaccenyl Acetate, A Female-Produced Sex Pheromone Component of *Ortholeptura valida*, A Longhorned Beetle in the Subfamily Lepturinae

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Abstract We report the identification, synthesis, and field bioassays of a female-produced sex attractant pheromone component of the cerambycid beetle *Ortholeptura valida* (LeConte). Headspace volatiles from females contained a female-specific compound, (*Z*)-11-octadecen-1-yl acetate, which elicited a strong response from antennae of adult males in coupled gas chromatography-electroantennogram analyses. In field bioassays, significant numbers of males were collected by traps baited with this compound. The pheromone represents a new structural class of cerambycid pheromones, and is the first pheromone identified for a cerambycid species in the subfamily Lepturinae.

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Key Words Cerambycidae · Sex pheromone · (*Z*)-11-Octadecen-1-yl acetate · *cis*-Vaccenyl acetate · Woodborer

Introduction

Cerambycid beetles of the subfamily Lepturinae (*sensu* Napp, 1994) are primarily Holarctic in distribution (Linsley, 1959; Švácha and Danilevsky, 1986), and the larvae typically develop in decaying wood (Linsley, 1959; Linsley and Chemsak, 1972; Švácha and Danilevsky, 1988). The adults of many species are diurnal and feed on the pollen or nectar of flowers (Linsley, 1961; Linsley and Chemsak, 1972). Other than those general habits, however, little is known of the behavior of most lepturine species (e.g., Swaine and Hopping, 1928; Michelsen, 1963; Linsley and Chemsak, 1972; Švácha and Danilevsky, 1988). We previously have suggested that mate location in lepturines is mediated by host plant volatiles (e.g., see Hanks, 1999; Ray, 2009). To our knowledge, long-range pheromones have not been reported for any species in the subfamily. Adult males of some lepturine species mate with females only after contacting them with their antennae (Michelsen, 1963), suggesting that mate recognition is mediated by contact pheromones (Millar et al., 2009).

We report here the identification, synthesis, and field bioassays of a female-produced sex attractant pheromone component of the lepturine *Ortholeptura valida* (LeConte). The active compound, (*Z*)-11-octadecen-1-yl acetate, represents the first example of a new structural class of pheromones within the Cerambycidae. *Ortholeptura valida* is endemic to the Pacific coast of North America, and

occurs from British Columbia to southern California along the Cascade, Sierra Nevada, Transverse, and Peninsular mountain ranges (Linsley and Chemsak, 1976; Švácha and Danilevsky, 1986, 1988). The larvae develop in the decaying trunks of conifers of many species (Craighead, 1923; Linsley and Chemsak, 1976; Cope, 1984; MacRae and Rice, 2007). Adults emerge from late June to August, and mate and oviposit on trees that are stressed, moribund, or have recently died (RLA, pers. obs.; Linsley and Chemsak, 1976). *Ortholeptura valida* is unusual among lepturines in that the adults are nocturnal (Linsley and Chemsak, 1976), and apparently do not feed on flowers (e.g., Švácha and Danilevsky, 1988). Adults often are collected at lights at night (Linsley and Chemsak, 1976).

Methods and Materials

Source of Beetles We collected prepupal larvae of *O. valida* on 3 June 2010 from downed *Abies concolor* Lind. & Gord. in the Barton Flats region of the San Bernardino National Forest (along California State Route 38, Mountaintop Ranger District, San Bernardino Co., CA, USA; N 34° 10′ 6″ W 116° 53′ 31″, 1940 m elevation). Dominant vegetation included mature *A. concolor*, *Pinus jeffreyi* Grev. & Balf., *P. ponderosa* Dougl. ex P. & C. Lawson, and *Quercus kelloggii* Newb. We extracted larvae from decomposing wood with an axe, and placed them in 25-ml plastic vials. In the laboratory, artificial pupation chambers were constructed from newsprint that was rolled into tubes (~7 cm long, ~2 cm diam). We placed a single larva in each tube, plugged the ends with tissue paper, misted them with water, and stored tubes together in a 4 l plastic bag at room temperature. Tubes were examined 2–3 times per week to monitor development.

The beetles eclosed during 20 June–5 July 2010. Teneral adults were returned to their paper tubes and temporarily stored at 4°C. Beetles were sexed using the sexually-dimorphic characters of antennal length and shape of the terminal abdominal sternite (Linsley and Chemsak, 1976). Two beetles of each sex were prepared for aeration by transferring them into individual vials (20 ml), and leaving them under ambient room conditions for 24 hr. The adults were virgins, and their cuticles were not fully sclerotized at the time that they were aerated.

Collection of Volatiles We collected headspace odors of adult male and female *O. valida* in an environmentally controlled room (26°C, 65% RH). Individual beetles were placed in modified 250 ml Ball® Mason-style canning jars that contained a glass vial (~2 ml) of 10% sucrose solution plugged with cotton, as nourishment. The jar lids were fitted with a Teflon® liner and two brass bulkhead unions (pipe thread, ~0.64 cm to 0.32 cm; Swagelok®, San Diego Valve and Fitting Co., San Diego CA, USA), one of which was connected to a flow meter-controlled vacuum source, and the other to a charcoal scrubber. Collectors were 6-mm OD by 41 mm long glass tubes containing a 5 mm long bed of thermally-desorbed activated charcoal (200 mesh; Fisher Scientific, Pittsburgh, PA, USA) held in place by Soxhlet-extracted (ether) glass wool plugs. The collectors were attached to jar lids with brass fittings (0.64 cm) with Teflon® ferrules (Swagelok®). Charcoal-filtered air was pulled through the chamber and collector at ~160 ml/min. Aerations were run for 4 days, after which the collectors were extracted with dichloromethane (3 rinses, for a total volume of 500 µl). Extracts were stored in a freezer (−4°C).

Coupled Gas Chromatography-Electroantennogram Detection (GC-EAD) and Gas Chromatography–Mass Spectrometry (GC-MS) Analyses GC-EAD analyses were performed using DB-5 and DB-Wax columns (both 30 m× 0.25 mm i.d., 0.25 µm film; J&W Scientific, Folsom, CA, USA). The carrier and makeup gas was helium. The GC oven was programmed from 40°C for 1 min, 10°C (5°C for calculation of Kovat's indices [KI] on DB-Wax) per min to 275°C for DB-5 and 250°C for DB-Wax, hold for 45 min. Solvent extracts (1 µl aliquots) were analyzed in splitless mode. The effluent from the columns was split with half of the sample going to the FID detector and the other half to the EAD. The portion directed to the EAD was diluted in a humidified air stream that was directed over the antennal preparation that consisted of the excised terminal 4–5 antennal segments of a male beetle, with the distal tip cut off with a razor blade, placed between two saline-filled glass capillary electrodes (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, and 0.20 g $NaHCO_3$ in 1 l Milli-Q® purified water). The glass electrodes were fitted with 0.2 mm diam gold wires that connected to the amplifier, with the amplifier gain set at 100X amplification. A single antennal preparation was used for as many as ten runs. GC-MS analyses were carried out with an Agilent 6890 N GC interfaced to a 5975 C mass selective detector (Agilent, Santa Clara, CA, USA). The GC was fitted with an HP5-MS column (30 m×0.25 mm i.d., 0.25 µm film), and the same temperature program and injection conditions were used as described above. KI were calculated for unknowns and standards relative to blends of straight-chain hydrocarbons. For increased precision, KI values on the DB-Wax column were obtained using a GC oven program rate of 5 rather than 10°C/min.

Epoxidation of the Unknown Approximately half of an aeration extract from a female beetle was treated with 20 µl of a solution of *m*-chloroperbenzoic acid in methylene chloride (2 mg/ml), and the solution was held at room temperature for 1 hr, swirling occasionally. The mixture was then concentrated just to dryness under a stream of

nitrogen, the residue was taken up in pentane, and loaded onto a small column of silica gel (2 cm×3 mm ID). The column was eluted successively with pentane (400 µl) and 2×300 µl 7.5% EtOAc in pentane. The resulting fractions were concentrated to ~25 µl and analyzed by GC-MS as described above. The epoxidized pheromone was in the third fraction.

Synthesis of (Z)*-11-Octadecen-1-yl acetate* Lithium aluminum hydride ($LiAlH_4$, 150 mg, 4 mmol) was added to 10 ml of dry THF under argon, and the mixture was cooled in an ice bath while a solution of *cis*-vaccenic acid (300 mg, 1.06 mmol; Sigma Chemical Co., St. Louis, MO, USA) in 3 ml THF was added dropwise. When the addition was complete, the ice bath was removed and the mixture was stirred overnight. The mixture was cooled in an ice bath and quenched by cautious addition of water (0.16 ml; foams!), 2 M NaOH (0.12 ml), and water (0.56 ml). The resulting slurry was stirred 15 min, during which time the inorganic salts precipitated out as a gummy white solid. The mixture was filtered through a short pad of Celite® (Fisher Scientific, Fairlawn, NJ, USA), rinsing the solids well with ether. The filtrate was dried over anhydrous Na_2SO_4, and then concentrated on a rotary evaporator. The resulting alcohol was taken up in a solution of 25 ml dry ether, and pyridine (160 µl, 2 mmol) and acetyl chloride (145 µl, 2 mmol) were added. The solution was stirred overnight at ~22°C, then poured into water and extracted with ether. The ether extract was washed with brine, dried over anhydrous Na_2SO_4, concentrated, and Kugelrohr distilled (oven temp. to 110°C, 0.05 mm), yielding (*Z*)-11-octadecen-1-yl acetate (270 mg, >99% pure by GC). The proton NMR spectrum was in agreement with that previously reported (Vig et al., 1988).

Field Bioassays of Synthetic Compounds We tested the response of adult *O. valida* to the female-specific component of the aeration, (*Z*)-11-octadecen-1-yl acetate, in field bioassays. We used black flight-intercept panel traps (1.2 high×0.3 m wide, model PT Intercept™, APTIV, Portland, OR, USA). Internal surfaces of traps were treated with Fluon®, which renders traps more slippery and can greatly improve trap catch of cerambycid beetles (Graham et al., 2010). To capture beetles alive, traps were modified by replacing the collection basin with a 1.9 l plastic funnel that was attached to a clear plastic jar (1.9 l, General Bottle Supply Company, Los Angeles, CA, USA). The funnel spout was cut off, leaving a 3.5-cm-diam hole at the bottom, a ~10 cm hole was cut into the threaded lid of the jar, and the funnel spout was hot-melt glued to the lid so that the spout was inside the jar. We hung traps from tree branches or from L-shaped hangers constructed of PVC pipe (as in Graham et al., 2010) with the trap bases ~30 cm above the ground. Pheromone lures were clear low-density polyethylene press-seal bags ("Zipper" seal sample bags, 2"×3", 51 µm wall thickness, #01-816-1A; Fisher Scientific, Pittsburgh, PA, USA) loaded with 25 mg of (*Z*)-11-octadecen-1-yl acetate in 475 µl of absolute ethanol (500 µl total volume) and sealed. Control lures contained 500 µl of absolute ethanol. Lures were suspended with wire in the central open area of traps.

We monitored flight activity of *O. valida* with two pheromone-baited sentinel traps at the Barton Flats study site. The sentinel traps were set up on 28 July 2010, within the seasonal activity period of the species (Linsley and Chemsak, 1976). Seven adult male *O. valida* were captured by sentinel traps within 2 days, and the field bioassay then was set up and run until 25 August 2010 (clear skies, no precipitation, air temperatures 10–33°C, maximum wind speed 11 km/h). We set up three replicates of the two treatments at the Barton Flats site, and two replicates at another site ~4 km to the southwest (along West Jenks Lake Road; N 34° 9′ 47″ W 116° 53′ 57″, 2020 m elevation). At both sites, traps were positioned in a southerly transect and separated by ~10 m, with pheromone and control treatments assigned randomly to traps.

Traps were checked for beetles at intervals of 4–6 days, and were shifted along transects every 7 days to control for location effects, at which time lures were replaced. Captured beetles were sexed using the sexually-dimorphic characters listed above. Differences between trap treatments in the number of beetles captured per trap per time interval were tested with the nonparametric Friedman's test (blocked by date and site; PROC FREQ with CMH option; SAS Institute, 1999) because assumptions of analysis of variance were violated by heteroscedasticity (Sokal and Rohlf, 1995). We excluded from the analysis one date on which no beetles were captured.

Voucher specimens of *O. valida* have been deposited at the Entomology Research Museum at the University of California, Riverside (museum voucher numbers: UCRC ENT 00282753, 00282754, 00282755, 00282756, 00282757, 00282758).

Results

Identification of the Pheromone Analyses of the extracts of volatiles released by female *O. valida* showed that a single sex-specific trace component in the extracts elicited a strong and reproducible response from antennae of male beetles (Fig. 1), with a KI on the relatively nonpolar DB-5 column of 2192 and a KI on the polar DB-Wax column of 2512. The EI mass spectrum showed a series of regularly spaced ion clusters separated by ~14 mass units, suggestive of a long hydrocarbon chain (Fig. 2, top). The values of the

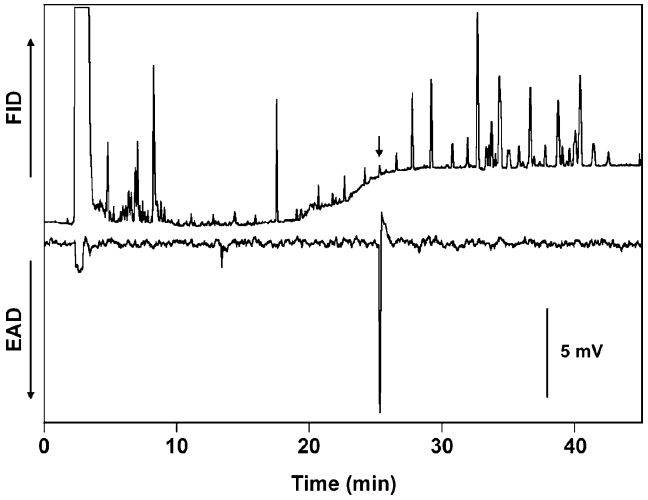


Fig. 1 Coupled gas chromatography-electroantennogram analysis of an extract of headspace odors from a female *Ortholeptura valida*. Upper trace is the chromatogram; lower inverted trace is the electroantennogram signal from the antenna of a male beetle. DB-5 column, 40°C/1 min, then 10°C/min to 275°C, hold 40 min. Scale bar represents 5 mV for the amplified antennal response (gain 100X)

most prominent ions in each cluster (*m/z* 69, 82, 96, 109, 123, etc.) suggested that the compound was unsaturated, or that it had a functional group that was readily lost as a neutral fragment to leave an unsaturated ion that fragmented further. A prominent fragment at *m/z* 61 suggested the presence of an acetate. The highest mass ion observed was at *m/z* 250 (possible formula, $C_{18}H_{34}$), which could reasonably be derived from the loss of acetic acid from a monounsaturated 18 carbon acetate ester.

GC-MS analyses of the model compound (*Z*)-9-octadecen-1-ol (oleyl alcohol) revealed that its mass spectrum was quite similar to that of the unknown, but the Kovats index (2062) on the DB-5 column was 130 units less than that of the unknown. However, a standard of (*Z*)-9-octadecen-1-yl acetate gave a retention index (2186) and a mass spectrum that was similar, but not an exact match to that of the unknown, indicating that the unknown was probably one of the other octadecenyl acetate isomers. The double bond position and geometry were determined by epoxidation of an extract, followed by GC-MS analysis. The resulting derivative gave enhanced fragments at *m/z* 127 ($C_8H_{15}O^+$) and 241 ($C_{14}H_{25}O_3^+$) from cleavage on either side of the epoxide, identifying the double bond in the parent compound as being in the 11 position (Fig. 3, top). An exact retention time match of the derivative with an epoxidized standard of (*Z*)-11-octadecen-1-yl acetate (vaccenyl acetate, Z11-18:Ac) indicated that the double bond in the unknown had the *Z*-configuration (*cis*- and *trans*-epoxides from *cis*- and *trans*-alkenes, respectively, have different retention times; Attygalle, 1998). This was confirmed by the presence of a trace amount of the corresponding *trans*-epoxide in the synthetic standard; the *cis*- and *trans*-epoxides were separated to baseline on the DB-5 column (*cis*: retention time 22.70 min; *trans*: 22.56 min). The epoxidized insect extract showed a peak only at 22.70 min, indicating that the insect produced only the *Z*-stereoisomer. The identification was confirmed by exact matches in retention times between the unknown and Z11-18:Ac on both the DB-5 and DB-WAX columns, and matching mass spectra (Fig. 3). Thus, the highest mass ion at *m/z* 250 observed in EI mass spectrum of the parent compound did indeed correspond to loss of acetic acid from the molecular ion (*m/z* 310), as commonly occurs with long-chain acetates under 70 eV electron impact ionization conditions.

Field Bioassays of Synthetic Compounds Traps baited with pheromone lures captured 139 and 41 male *O. valida* (a total of 180 beetles) at the Barton Flats and Jenks Road sites, respectively, but no beetles were captured by control traps. As many as 46 male beetles were captured per

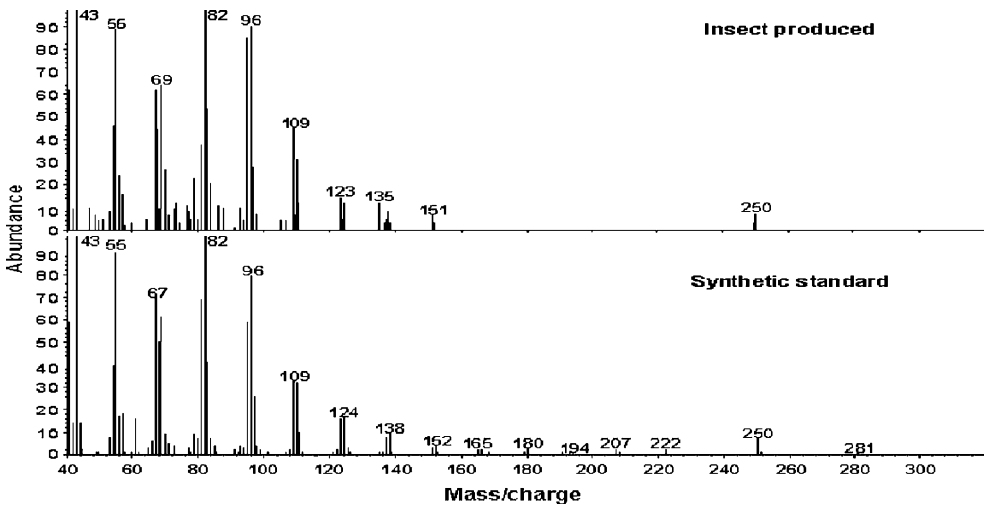


Fig. 2 EI mass spectra (70 eV) of the compound that elicited a strong electroantennogram response in Fig. 1 (top) and of an authentic standard of (*Z*)-11-octadecen-1-yl acetate

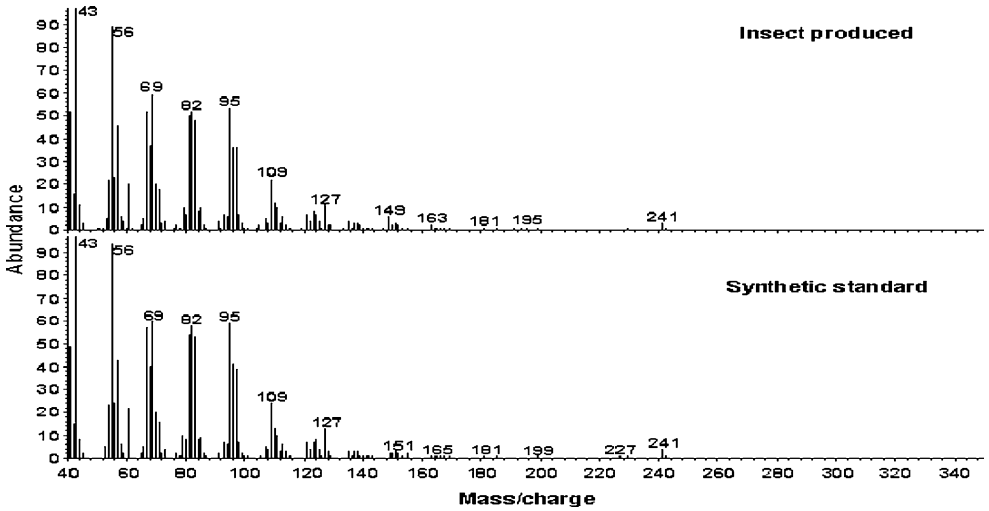


Fig. 3 EI mass spectra (70 eV) of the epoxide of the compound that elicited a strong EAD response in Fig. 1 (top) and of epoxidized authentic (*Z*)-11-octadecen-1-yl acetate

pheromone trap per date, with a mean of 7.5±1.9 beetles (SE; significantly different from the 0 mean for controls; Friedman's $Q_{1,48}$=28.7, P<0.001). Traps baited with the synthetic pheromone did not capture any other insect species.

Discussion

Exclusive attraction of *O. valida* males to lures baited with synthetic (*Z*)-11-octadecen-1-yl acetate indicates that this compound is a sex pheromone for this species. The strong attraction in field bioassays, coupled with the lack of any other significant responses from antennae of male beetles in GC-EAD analyses of aeration extracts of females, strongly suggests that the pheromone consists of only this single component. Female-produced pheromones also have been found in species of the cerambycid subfamily Prioninae (Rodstein et al., 2011), and species that apparently are related to the Cerambycidae, including *Migdolus fryanus* Westwood (Anoplodermatinae; Leal et al., 1994) and *Vesperus xatarti* Dufour (Vesperidae; Boyer et al., 1997; for phylogenetic relationships, see Švácha and Danilevsky, 1986; Napp, 1994). Females of those species generally are larger than males and sedentary, being incapable of flight until after they have oviposited, permanently incapable of flight, or at least characteristically reluctant to fly (Linsley, 1959). The sedentary behavior of the females is consistent with the persistent nature of the larval hosts: living roots of perennial grasses, shrubs, or trees (e.g., Leal et al., 1994; Barbour et al., 2006). Individual hosts, therefore, can support many generations, and females need not disperse to locate appropriate new hosts (Hanks, 1999). Newly eclosed females of these species may remain concealed in the soil, or within tissues of the larval host, and release pheromones that attract their mates (Leal et al., 1994; Hanks, 1999; Barbour et al., 2006). The early release of pheromone by females, and strong response of males, suggests an urgency that is consistent with limited energy reserves due to the fact that the adults likely do not feed (Linsley, 1959; Hanks, 1999; Barbour et al., 2006).

In contrast to other species that have female-produced pheromones, the larvae of *O. valida* develop in hosts that are dying, or have recently died. Such hosts are unpredictable and ephemeral resources, since their tissues degrade rapidly (reviewed by Hanks, 1999). Therefore, adult females must be capable of dispersing to locate appropriate hosts, which is consistent with reports that they take flight readily (Linsley and Chemsak, 1976). This relationship between larval host condition and dispersal ability in adults is more consistent with species in the subfamilies Cerambycinae, Lamiinae, and Spondylidinae in which males produce pheromones that attract both sexes (Silk et al., 2007; Millar et al., 2009; Ray et al., 2009; Fonseca et al., 2010; Pajares et al., 2010). Larvae of those species also develop in highly stressed or moribund woody plants, and the pheromones apparently serve to expedite location of hosts (Hanks, 1999).

The structure of the *O. valida* pheromone is unprecedented among the cerambycid pheromones that have been reported to date, and completely unlike the diol/hydroxyketone motif of the volatile pheromones produced by species in the subfamily Cerambycinae (Millar et al., 2009), the terpene motif of the pheromones produced by males of species in the subfamily Spondylidinae (Silk et al., 2007), or the carboxylic acid motif of species in the genus *Prionus* (subfamily Prioninae; Rodstein et al., 2011). (*Z*)-11-Octadecen-1-yl acetate also is an aggregation pheromone produced by male fruit flies (*Drosophila* spp.; Bartelt et al., 1985), but when transferred to females during mating it subsequently discourages courtship of other males (Jallon et al., 1981). This compound also has been reported as a minor

component in the defensive secretions of larvae of the chrysomelid beetle *Linaeidea aenea* (L.) (Sugawara et al., 1979). In addition, (*Z*)-11-octadecen-1-yl acetate is similar in structure to the monounsaturated acetate compounds that are pheromone components of many lepidopterans (El-Sayed, 2011).

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Sex Pheromone and Trail Pheromone of the Sand Termite *Psammotermes hybostoma*

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Abstract Within the complex network of chemical signals used by termites, trail pheromones and sex pheromones are among the best known. Numerous recent papers map the chemical identity and glandular origin of these pheromones in nearly all major isopteran taxa. In this study, we aimed to describe the sex pheromone and the trail pheromone of a poorly known sand termite, *Psammotermes hybostoma*. We identified (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (dodecatrienol) as the sex pheromone released by tergal and sternal glands of female imagos and, at the same time, as the trail pheromone secreted from the sternal gland of workers. We conclude that chemical communication in *Psammotermes* does not differ from that of most other Rhinotermitidae, such as *Reticulitermes*, despite the presence of a diterpene as a major component of the trail pheromone of *Prorhinotermes* to which *Psammotermes* is presumed to be phylogenetically close. Our findings underline once again the conservative nature of chemical communication in termites, with dodecatrienol being a frequent component of pheromonal signals in trail following and sex attraction and, at the same time, a tight evolutionary relationship between the trail following of working castes and the sex attraction of imagos.

Key Words Sex pheromone · Trail pheromone · *Psammotermes hybostoma* · Termites · Rhinotermitidae · Tergal glands · Sternal gland · GC-EAD · Dodecatrienol · Timber pest

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Introduction

Devoid of visual orientation, termites have reached a high level of social and functional complexity, with semiochemicals being involved in many aspects of their life. Within the network of chemical signals used by termites, the trail pheromones and the sex pheromones are among the best known. Numerous recent papers map the chemical identity and glandular origin of these pheromones in all but one (Serritermitidae) of the extant isopteran families using modern methods of chemical extraction (solid phase microextraction) and electrophysiological experiments (Sillam-Dussès et al., 2009; reviewed in Bordereau and Pasteels, 2011).

This progress in chemical ecology allows us to discern several particularities of termite trail and sex pheromones, contrasting with the situation in social hymenopterans. First, only a few glands are involved in the trail-following communication and sexual attraction in termites. Trail pheromones are always produced by the same homologous gland, the sternal gland of workers (but the gland is present in all castes and developmental stages) (Quennedey et al., 2008). The sex pheromones are secreted from the sternal gland and/or the tergal glands and/or the posterior sternal glands of female, or rarely from male imagoes or both sexes (reviewed in Pasteels and Bordereau, 1998; Bordereau and Pasteels, 2011). Second, the chemical diversity of pheromonal components is surprisingly low, with only 8 compounds being identified as trail pheromones in more than 60 species studied, and only 9 compounds confirmed so far to participate in sexual attraction in 25 species studied

(Bordereau and Pasteels, 2011). Third, along with the low diversity in anatomy and chemistry, there is striking parsimony in the pheromones of the many species observed, with the same compound acting as trail and sex pheromone though in different concentrations and in different contexts (reviewed in Pasteels and Bordereau, 1998; Bordereau and Pasteels, 2011). Fourth, both trail and sex pheromones most often consist of a single compound, not blends of compounds as in numerous other insects. However, some recent studies demonstrated the presence of minor components in addition to these major compounds (Sillam-Dussès et al., 2009, 2010; Bordereau et al., 2010; Anani Kotoklo et al., 2010), suggesting that minor active components could have been overlooked in the past.

Nevertheless, despite the conservative nature of both trail and sex pheromones and their glandular origins, their chemical ecology still represents a useful comparative tool at higher taxonomical levels. It is particularly true for the situation within the paraphyletic family Rhinotermitidae, within which both Serritermitidae and Termitidae probably evolved (see recent molecular phylogenetic studies, e.g., Lo et al., 2004; Ohkuma et al., 2004; Inward et al., 2007; Legendre et al., 2008). Most rhinotermitids studied so far use the unsaturated alcohol (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (dodecatrienol) as their trail pheromone (e.g., Matsumura et al., 1968; Tai et al., 1969; Tokoro et al., 1989, 1991; Wobst et al., 1999; Sillam-Dussès, 2004), and also as the sex pheromone produced by the sternal or tergal glands of females (Laduguie et al., 1994a,b; Hanus et al., 2009). However, the subfamily Prorhinotermitinae displays another combination of glands and compounds, similar to the situation in Termitidae (Bordereau et al., 2002, 2010; Sillam-Dussès et al., 2010): the trail pheromone consists of the diterpene neocembrene (major component) and dodecatrienol (Sillam-Dussès et al., 2009); the sex pheromone is once again dodecatrienol, but it is secreted by female tergal glands (Hanus et al., 2009). This striking singularity of *Prorhinotermes* promoted our interest in the chemistry of sex and trail pheromones in other basal rhinotermitid genera, namely *Psammotermes*, which is suggested to be a sister clade to *Prorhinotermes* in several recent phylogenies (Austin et al., 2004; Lo et al., 2004; Ohkuma et al., 2004; Inward et al., 2007).

Species in the genus *Psammotermes*, Desneux, 1902, which are important timber pests, inhabit arid areas of the Old World, namely Sahara and Arabia (*P. hybostoma* Desneux, 1902), South Africa (*P. allocerus* Silvestri, 1908), Madagascar (*P. voeltzkowi* Wasmann, 1910), and South-West Asia (*P. rajasthanicus* Roonwal & Bose, 1960). However, the most basic aspects of *Psammotermes* biology remain unknown, such as the caste system (only a pronounced size polymorphism of workers and soldiers has been noted; Clément, 1952; Roonwal, 1988) or the chemistry of the defensive frontal gland of soldiers. Together with Stylotermitinae and Termitogetoninae, Psammotermitinae is among the last rhinotermitid subfamilies that have not yet been studied with respect to the trail and sex pheromones. In this study, we report the results of our investigations on the source and chemical identity of the trail pheromone and the sex pheromone in the sand termite *Psammotermes hybostoma*.

Methods and Materials

Insects Large portions of *Psammotermes hybostoma* colonies were collected from tamarisk wood at the periphery of Sohag, Egypt and brought to Prague, Czech Republic. The colonies were held under laboratory conditions in their original wood at 26°C and a low relative humidity. Some of the colonies contained alate imagos of both sexes waiting for their dispersal flight. These were kept in the colonies and used in the study of the sex pheromone. Males were eventually dealated prior to behavioral experiments. The readiness of males to respond to female sex pheromone has been tested by observation of tandem behavior. Within the polymorphic worker caste, the "large workers" represented a minority of individuals, their number being insufficient for the purpose of this study. Moreover, Clément (1952) questioned the participation of "large workers" in colony tasks. Therefore, only individuals that could be classified as "minor workers" were included in trail pheromone analysis.

Histology of Exocrine Glands Semithin resin sections were prepared with male and female dealate imagos and with workers (see Šobotník et al. (2010) for details of fixation procedure). Sections (1 μm) were cut using an Ultracut Reichert-Jung, stained with Azur II solution, and studied using a Carl Zeiss Amplival optical microscope combined with a Canon EOS 500D camera.

Pheromone Standard (3*Z*,6*Z*,8*E*)-Dodeca-3,6,8-trien-1-ol (dodecatrienol) was kindly supplied by Christian Bordereau (Dijon, France).

Glandular Extracts The following hexane extracts were prepared after dissection of females and workers: 250 sternal glands of workers, 500 sternal glands and 500 tergal glands of female imagos, 500 female bodies devoid of the sternal and tergal glands. Extracts were diluted with hexane to two concentrations, i.e., 1 and 10 gland equivalents per 10 μl. These extracts were used in the sex attraction bioassays, and trail-following bioassays. Concentrated glandular extracts were used for electrophysiological experiments and gas chromatography (GC).

Electrophysiological Experiments Electroantennographic bioassays (EAG) and gas chromatography coupled with electroantennographic detection (GC-EAD) were performed with antennae of males and workers as described in Sillam-Dussès et al. (2009). Concentrated extracts of sternal and tergal glands of female imagos, concentrated extracts of sternal glands of workers, and a solution of a standard of dodecatrienol were used in GC-EAD. The applied solutions were co-eluted with a series of *n*-alkanes (C8-C22) in order to calculate the linear retention indices (LRI) of particular compounds. A DB-5 column (30 m×20.25 mm i.d., 0.25 μm film; J&WScientific, Folsom, CA, USA) was used, programmed from 40°C (2 min) to 270°C (10 min) at 50°C/min. Injector and detector temperatures were 220°C and 250°C, respectively. Glandular extracts and dodecatrienol (0.1–100 pg per stimulation) were tested in EAG, with hexane and air being used as controls.

Gas Chromatography The extract of tergal and sternal glands of females, and sternal glands of workers were analyzed by two-dimensional GC with mass spectrometric time of flight detection (GC × GC/TOF-MS, Pegasus 3D, Leco, St. Joseph, MI, USA). A nonpolar DB-5 column as above was used in the first column, and a polar BPX-50 column (2 m×0.1 mm i.d, 0.1 μm film; SGE, Melbourne, Australia) was used as the second column. The first GC was programmed from 40°C (1 min) to 320°C at 7°C/min, and the second column was set at 340°C. 1 μl Samples were injected in a splitless mode; injector temperature was 250°C; and helium (1.0 ml/min) was used as carrier gas. Separation time in the second dimension was 4 sec with a 0.6 sec hot pulse. Detector conditions: The EI source for the TOF-MS operated at temperature 220°C using 70 eV ionization, and an acquisition rate 50 spectra/s.

Sex Attraction Choice Bioassays Two dealate male imagos were introduced at the same time in a 15 cm Petri dish lined with Whatman No. 1 filter paper. The dish contained two folded pieces of filter paper (1 cm^2), treated with 10 μl of hexane extract of female bodies (devoid of sternal and tergal glands) or sternal and/or tergal glands extracts of females (always 1 female equivalent per paper) or various concentrations of dodecatrienol standard or 10 μl of hexane as control. Two males were used because a single male deprived of tactile communication displays an unstable behavior. The time spent by one or two males in contact with particular pieces of paper was measured during 5 min. Results obtained from 15 replicates for each treatment combination were statistically analyzed by using Mann-Whitney *U* test.

Trail-following Choice Bioassays Trail-following bioassays were performed to test the orientation activity of hexane extracts of sternal glands. Sternal gland extracts of females were used in tests with males, worker sternal gland extracts in tests with workers, and dodecatrienol standard solutions. Solutions were tested with a 'Y open field' bioassay on Whatman No.1 filter paper discs (15 cm diam), with a 120° angle between each branch. On the stem (3 cm) and one of the branches (7 cm), an artificial trail was drawn with a syringe containing 10 μl of sternal gland extract (1 μl/cm of the trail, i.e., 0.1 or 1 gland equivalent per cm of the trail). Ten μl of hexane were deposited on the stem and the other branch of the Y. One termite was placed inside a holding chamber (55 mm Petri dish), with a 2-mm opening located at the base of the Y. The distance travelled by the insect on the trail was measured. The activity threshold was defined as the minimum concentration that elicited termites to travel a mean distance of more than 3 cm, the maximum response being 10 cm. Each experiment was repeated with 15 or 30 individual termites. Choice tests were performed with the same experimental setup to compare the preference of termites between gland extracts and dodecatrienol. Each of the compared stimuli was deposited on the stem and on one of the Y branches of the experimental arena. This test was repeated with 30 individual termites, and results were evaluated using χ^2-test.

Results

Origin of the Female Sex Pheromone – Microscopy Semi-thin sections of *P. hybostoma* female imagos revealed the presence of three well developed tergal glands in the anterior parts of the 8th, 9th, and 10th tergites, and of one sternal gland in the anterior part of the 5th sternite (Fig. 1). Tergal glands consist of two common cell types, and these are probably class 1 and class 3 secretory cells (*sensu* Noirot and Quennedey, 1974, 1991). Tergal glands are between 130 and 180 μm long, and up to 50 μm thick. We did not observe the tergal glands in *P. hybostoma* males. The sternal gland of imagos is about 250 μm long and 35 μm thick, and its external reservoir is quite large (see Fig. 1).

Origin of the Female Sex Pheromone – Behavior Calling behavior was not observed in either sex in the field or the laboratory, but tandems (a female followed by a male) were formed immediately after encountering the opposite sex (Fig. 2). In sex attraction bioassays, males preferred the mixed extract of sternal and tergal glands of females to the extracts of whole female body devoid of these glands (63± 16 sec vs. 3±1 sec, $P<10^{-5}$). Males clearly preferred the extract of sternal glands and, even more, the extract of tergal glands of females to hexane controls (Table 1). No significant preference was observed when the extract of

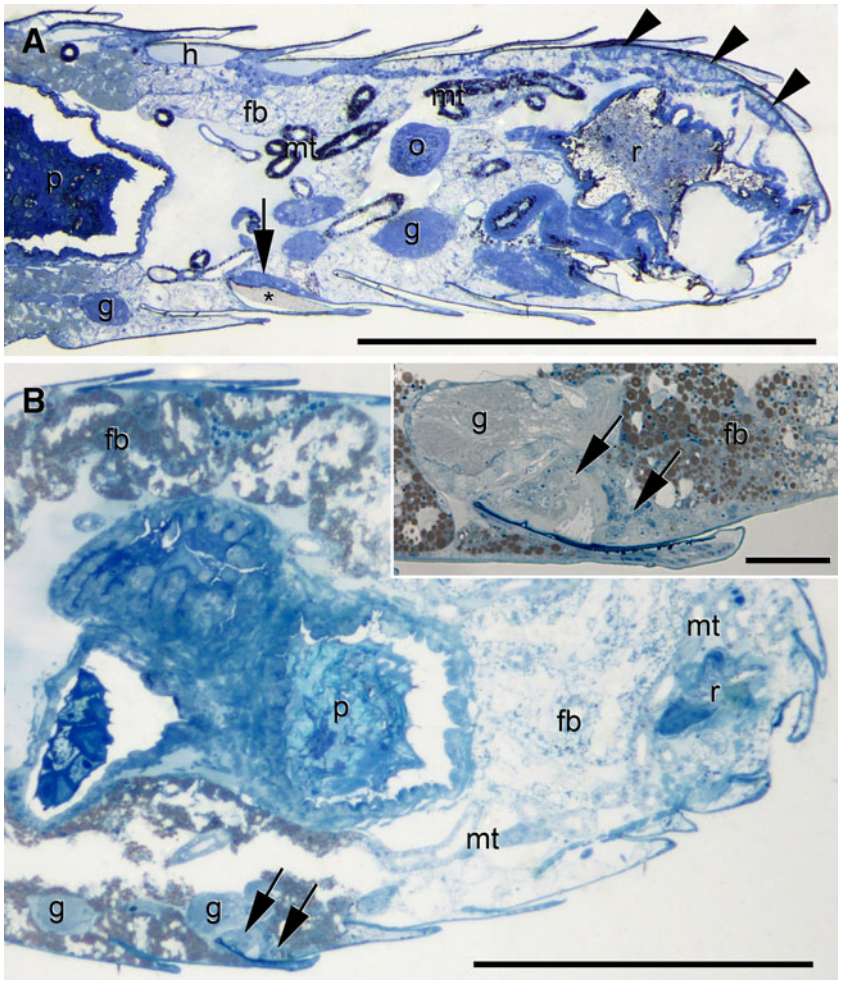


Fig. 1 Structure of exocrine glands involved in sexual attraction and trail following. **A**. Sagittal section of posterior abdomen of a female imago. Arrow marks the sternal gland; asterisk marks the external reservoir of the sternal gland; arrowheads mark three tergal glands. Scale bar represents 1 mm. **B**. Sagittal section of the posterior abdomen of a worker. Inset provides a detailed view on the sternal gland. Arrows mark the sternal gland. Scale bar represents 0.5 mm (50 μm in inset). Abbreviations: fb, fat body; g, ganglion of neural cord; h, heart; mt, malpighian tubules; o, oocyte; p, paunch; r, rectum

tergal glands and the mixed extract of tergal and sternal glands were exposed to the males (72±18 sec vs. 54±9 sec, N=15, P=0.56). In addition, trail-following bioassays showed that males were able to consistently follow the trails made with sternal gland extract of females (Table 2). All these results indicate that the components of the female sex pheromone are located in both the sternal gland and the tergal glands of female imagos.

Chemical Nature of the Female Sex Pheromone In GC-EAD experiments, injections of extracts of 6 female tergal glands elicited a single significant and reproducible major antennal response in males to a compound eluting at 7.21 min (Fig. 3a). Co-injection of the extract with a series of hydrocarbons revealed that the LRI of the putative active compound was approximately 1525. The retention index was characteristic for dodecatrienol. Indeed, the injection of 10 ng of dodecatrienol resulted in a clear FID peak at the same retention time, and a corresponding antennal response (Fig. 3c). Subsequently, the presence of dodecatrienol in the tergal glands was confirmed by an analysis of the extract (25 gland equivalents) by GC × GC/TOF-MS.

The extract of female sternal glands elicited a single antennal response of males at the retention time identical to that observed for the tergal gland extract and dodecatrienol standard (Fig. 3b). Analysis of this extract (25 gland equivalents) by GC × GC/TOF-MS confirmed the presence of the dodecatrienol in the sample. We concluded that dodecatrienol is a component of the female sex pheromone of *P. hybostoma*, secreted by sternal and tergal glands.



Fig. 2 Imagos of *Psammotermes hybostoma* during the tandem run; a female followed by a male

Biological Activity of Dodecatrienol Sex attraction bioassays showed that males are highly attracted to dodecatrienol at 10 pg per paper and higher (Table 1). In the

Table 1 Sex attraction bioassays with dodecatrienol or female gland extracts[a]

Hexane	Tergal gland extract	Sternal gland extract	Dodecatrienol (pg/paper)						*N*	*P*
	1 TGE/paper	1 SGE/paper	1	10	100	10^3	10^4	10^5		
13±3	110±22	–	–	–	–	–	–	–	15	$< 10^{-3}$
16±3	–	58±17	–	–	–	–	–	–	15	< 0.05
9±2	–	–	14±3	–	–	–	–	–	15	*NS*
6±1	–	–	–	39±5	–	–	–	–	15	$< 10^{-3}$
6±3	–	–	–	–	68±16	–	–	–	15	$< 10^{-3}$
6±1	–	–	–	–	–	43±9			15	$< 10^{-3}$
8±1	–	–	–	–	–	–	110±13		15	$< 10^{-3}$
8±1	–	–	–	–	–	–	–	154±23	15	$< 10^{-3}$
–	57±21	–	–	24±4	–	–	–	–	15	*NS*
–	11±4	–	–	–	47±17	–	–	–	15	$< 10^{-3}$
–	15±4	–	–	–	–	80±18	–	–	15	$< 10^{-3}$
–	17±3	–	–	–	–	–	75±11	–	15	$< 10^{-3}$
–	9±2	–	–	–	–	–	–	92±14	15	$< 10^{-3}$

[a] The time spent by two dealate male imagos on papers impregnated with hexane, female tergal gland extract, female sternal gland extract, or dodecatrienol at 6 different concentrations was measured (mean in sec ± *S.E.M.*). TGE – tergal gland equivalent, SGE – sternal gland equivalent, *N* – number of repetitions, *P* value (Mann-Whitney *U* test), *NS* – non-significant.

choice test, males preferred dodecatrienol at 100 pg/paper and higher to tergal glands extract; this indicates that approximately 10 pg of dodecatrienol are present in the tergal glands of one female (Table 1).

In trail-following bioassays, the trail-following activity of males was elicited by dodecatrienol at a concentration 10 pg/cm and higher (Table 2). In the choice trail-following test, males preferred to follow the trail made with dodecatrienol at 100 pg/cm and higher to the extracts of sternal gland (1 gland equivalent per cm). This indicates that between 10 and 100 pg of dodecatrienol are present in the sternal gland of one female (Table 3).

Quantification of the Female Sex Pheromone In addition to the sex attraction and trail-following bioassays (see above), the dose-response relationship of the EAG response to dodecatrienol standard solutions compared to glandular extracts was used to estimate the quantity of dodecatrienol in sternal and tergal glands of females. EAG amplitude increased with increasing concentrations of dodecatrienol; dodecatrienol was estimated to be approximately 1 pg in the sternal gland, and 10 pg in tergal glands of one female (Fig. 4). The results for the two methods used are summarized and compared in Table 5.

Origin of the Trail Pheromone of Workers – Microscopy The presence of a single sternal gland located at the anterior part of the 5th sternite of worker was confirmed, as in all Rhinotermitidae studied so far (Fig. 1). The gland is approximately 170 μm long and 50 μm thick. It does not differ in overall size from the gland of female imagos, but its shape is bilobed, as in workers of other rhinotermitids (see Ampion and Quennedey, 1981).

Origin of the Trail Pheromone of Workers – Behavior - Workers followed trails made of the sternal gland extract of workers at both concentrations tested (5.1±1 cm at 10^{-1} gland equivalent/cm, *N*=15; 6.9±0.7 cm at 1 gland

Table 2 Trail-following bioassays with dodecatrienol or female sternal gland extracts[a]

Hexane	Sternal gland extract (SGE/cm)		Dodecatrienol (pg/cm)				*N*
	10^{-1}	1	1	10	100	1000	
0	1.9±0.6	–	–	–	–	–	15
0	–	5.7±0.85	–	–	–	–	15
0	–	–	2.1±0.4	–	–	–	30
0	–	–	–	3.7±0.6	–	–	30
0	–	–	–	–	8.5±0.5	–	30
0	–	–	–	–	–	9.7±0.3	30

[a] The distance travelled by one dealate male imago on trails made of sternal gland extract or dodecatrienol was measured (mean in cm ± *S.E.M.*). SGE – sternal gland equivalent, *N* – number of repetitions.

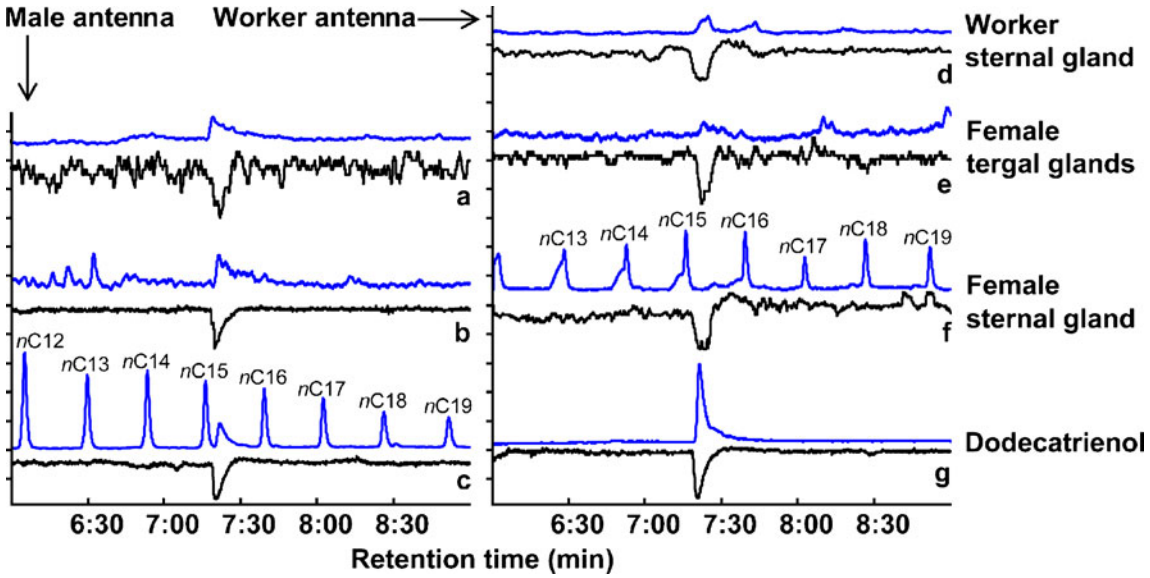


Fig. 3 Electroantennographic recordings of male (left) and worker (right) antennae exposed to the extracts of female tergal and sternal glands, worker sternal glands, and dodecatrienol standard solution. Upper curve (blue) – FID, lower curve (black) – EAD. Note the antennal response of both, males and workers, at the retention time 7.21 in all experiments. In c and f, the tested solutions are co-eluted with *n*-alkane standards (C8–22). *Y* scale (mV per division): a – EAD=0.1, FID=4; b – EAD=1, FID=3; c – EAD=1, FID=25; d – EAD=1, FID=5; e – EAD=0.1, FID=4; f – EAD=0.5, FID=4; g – EAD=1, FID=10

equivalent/cm, N=30). Therefore, we concluded that the trail pheromone of workers is secreted by their sternal gland.

Chemical Nature of the Trail Pheromone of Workers Injection of concentrated extract of sternal glands from 5 workers into the GC-EAD setup resulted in a single antennal response of workers, matching the retention characteristics of dodecatrienol as described above (Fig. 3d, g). The workers responded identically to the extracts of the female sternal and tergal glands, suggesting the presence of the same active component, dodecatrienol, in the glands (Fig. 3e, f). In addition, workers followed trails made of the tergal gland extract of female imagos (10^{-1} female tergal gland equivalent per cm), which confirms the identity of the female sex pheromone and the worker trail pheromone. The presence of dodecatrienol in the sternal gland extract of workers was confirmed using GC × GC/TOF-MS (50 gland equivalents injected). Thus, we concluded that dodecatrienol is a component of the trail pheromone of *P. hybostoma* workers.

Table 3 Trail-following choice bioassays to female sternal gland extract and dodecatrienol[a]

Sternal gland extract	Dodecatrienol (pg/cm)			*N*	*P*
1 SGE/cm	10	100	1000		
24	6	–	–	30	$< 10^{-3}$
4	–	26	–	30	$< 10^{-3}$
6	–	–	24	30	$< 10^{-3}$

[a] Values indicate the number of choices made by one dealate male imago when given a choice between a trail made of female sternal gland extract or dodecatrienol. SGE – sternal gland equivalent, *N* – number of repetitions, *P* value (χ^2-test).

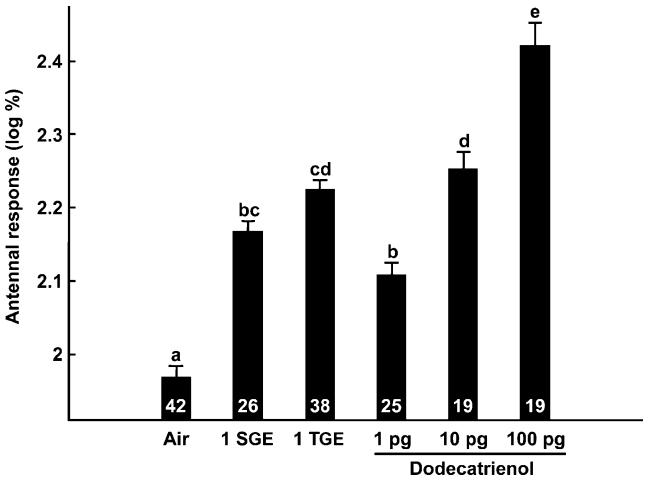


Fig. 4 EAG responses of males to air control, glandular extracts of females, and various concentrations of dodecatrienol (mean ± *S.E.M.*). Data were normalized to hexane stimulations and log transformed to reduce heteroscedasceity. Data normality was controlled using Levene's test (P=0.09). Bars marked with different letters indicate significant differences (P<0.05) among treatments, calculated by means of ANOVA ($F_{(5,169)}$=74.5, $P<10^{-4}$) with subsequent post-hoc comparison using HSD for unequal *N*. Numbers at the base of each bar represent the number of observations for each treatment. SGE = sternal gland equivalent; TGE = tergal gland equivalent

Table 4 Trail-following bioassays with dodecatrienol[a]

Dodecatrienol (pg/cm)					*N*
10^{-1}	1	10	100	1000	
1.1±0.2	–	–	–	–	30
–	3.8±0.4	–	–	–	30
–	–	5.8±0.6	–	–	30
–	–	–	3.4±0.6	–	30
–	–	–	–	2.9±0.5	30

[a] The distance travelled by one worker on trails made of dodecatrienol is measured. Another trail, made of hexane, is used as a control and is never followed. *N* – number of repetitions.

Biological Activity of Dodecatrienol Dodecatrienol was highly active in eliciting the trail-following of workers; the activity threshold being 1 pg/cm, with maximum activity observed at 10 pg/cm. A decrease in the trail-following activity was observed at 100 pg/cm and higher (Table 4). In the choice trail-following test, workers preferred dodecatrienol at a concentration 100 pg/cm to sternal gland extract (1 gland equivalent per cm), which suggests that approximately 10 pg are present in one gland.

Quantification of the Trail Pheromone of Workers In addition to the trail-following choice bioassays (see above), the EAG dose-response experiments were performed to estimate the quantity of dodecatrienol in sternal glands of workers. The threshold activity was observed at 1 pg of dodecatrienol; the quantity of dodecatrienol in the sternal gland of one worker was estimated to be between 1 and 10 pg (Fig. 5). The results of the two approaches are compared in Table 5.

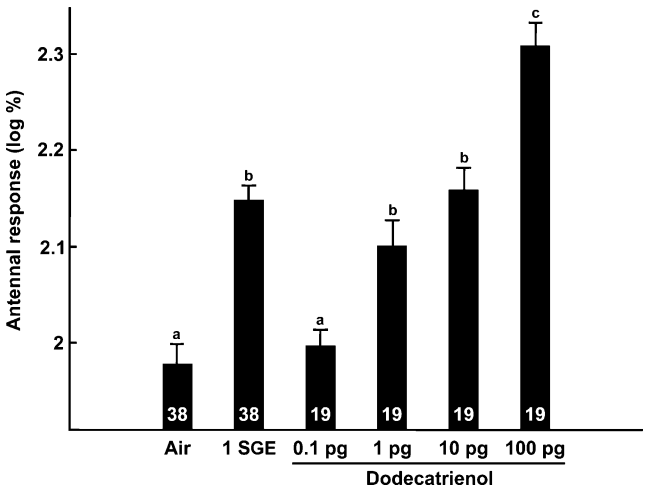


Fig. 5 EAG responses of workers to air control, sternal gland extract of workers, and various concentrations of dodecatrienol (mean ± *S.E.M.*). Data were normalized to hexane stimulations and log transformed to reduce heteroscedasceity. Data normality was controlled using Levene's test (P=0.075). Bars marked with different letters indicate significant differences (P<0.05) among treatments, calculated by means of ANOVA ($F_{(5,\ 152)}$=30.8, $P<10^{-4}$) with subsequent post-hoc comparison using HSD for unequal *N*. Numbers at the base of each bar represent the number of observations for each treatment. SGE = sternal gland equivalent

Discussion

Our study demonstrates that (3*Z*,6*Z*,8*E*)-dodeca-3,6,8-trien-1-ol (dodecatrienol) is the major component of the sex pheromone of female imagos and of the trail pheromone of workers in the sand termite *Psammotermes hybostoma*. The female sex pheromone is produced by the tergal glands and the sternal gland, and the trail pheromone is secreted by the sternal gland of workers. The identity of the two pheromones has been studied and confirmed using a combination of behavioral bioassays, electrophysiology, and chemical analysis.

For the first time, a termite sex pheromone secreted by two different glandular sources, the tergal glands and the sternal gland, has been chemically characterized. Previous studies implicated multiple glands in termite sexual attraction, such as the sternal gland and the tergal glands in *Trinervitermes bettonianus* (Termitidae: Nasutitermitinae) (Leuthold, 1975, 1977) and *Kalotermes flavicollis* (Kalotermitidae) (Wall, 1971), or the tergal and the posterior sternal glands in *Macrotermes annandalei* and *M. barneyi* (Termitidae: Macrotermitinae) (Peppuy et al., 2004), but the nature of the sex pheromones has not been identified in these species. The participation of the female tergal glands in the sexual attraction was obvious from their occurrence in histological sections; the female imagos being the only caste and sex with these glands present (see also Ampion and Quennedey, 1981). The role of the sternal gland and its secretion was less obvious but also likely, based on their histology: the gland is smaller than in *Reticulitermes* (Rhinotermitidae: Heterotermitinae), in which it is the sole source of the sex pheromone, but at the same time it is significantly larger than in the related *Prorhinotermes*, in which it does not participate in the sexual attraction and is vestigial (Šobotník and Hubert, 2003; Hanus et al., 2009).

Table 5 Comparison of the quantity of dodecatrienol in exocrine glands involved in sex attraction and trail following based on EAG experiments and behavioral bioassays

Caste	Gland	Dodecatrienol (pg)	
		EAG	Behavioral bioassay
Female imago	Tergal	10	10
Female imago	Sternal	1	10–100
Worker	Sternal	1–10	10

What would be the purpose of having two types of glands secreting the same compound for sexual attraction? Courtship in termites usually consists of calling behavior and tandem behavior, both associated with secretion of a sex pheromone. In *Psammotermes*, we did not observe the typical calling posture of females with the tergal glands exposed (see e.g., Hanus et al., 2009). However, the readiness of females to call might have been affected by the laboratory conditions. Alternatively, the females can secrete their sex pheromone without the characteristic calling posture. Nevertheless, the males are attracted by the females, and immediately after an encounter they form the typical tandem following the female tergal region with their heads and antennae. Therefore, we presume that females use their tergal glands to attract males at a short distance and to ensure the tandem cohesion, while they lay an odor trail with their sternal gland that prevents an accidental tandem separation, as described in other termite species (Nutting, 1969; Bordereau and Pasteels, 2011). Thus, interestingly, the same compound, dodecatrienol, would be used as a short-range airborne sex pheromone and as a trail pheromone. Nevertheless, although dodecatrienol was the only active compound identified, we cannot exclude that other, undetected compounds are involved.

Dodecatrienol has been confirmed as the major or only component of the sex pheromone in numerous termite species; it is produced by the tergal glands in Syntermitinae (Bordereau et al., 2002), and by the sternal gland in the macrotermitine *Pseudacanthotermes* (Bordereau et al., 1991) and in the rhinotermitid *Reticulitermes* (Laduguie et al., 1994a). In *Prorhinotermes*, probably the closest relative of *Psammotermes*, we also have recently ascribed the function of sex pheromone to dodecatrienol secreted from the tergal glands of females (but not from the vestigial sternal gland), as evidenced by bioassays and GC-EAD experiments (Hanus et al., 2009). However, we were not able to detect the compound using GC-MS, probably due to its very low concentrations in the tergal glands (units of picograms). In *Psammotermes* females, the content of the compound in the sternal gland and the tergal glands, and its active concentrations, also are very low (picograms) (see Table 5). Such low concentrations of dodecatrienol also have been observed in *Reticulitermes* (Laduguie et al., 1994a), and contrast with usually high concentrations of sex pheromones in other species (see Bordereau and Pasteels, 2011).

We also have demonstrated that dodecatrienol, secreted from the sternal gland of workers, is the trail pheromone used to mark foraging trails. Even though we cannot exclude the presence of multiple components, we observed no GC-EAD evidence of other compounds being involved in trail following. This finding is not surprising given that the sternal gland is a universal source of trail pheromones in termites, and dodecatrienol is used as a trail pheromone in all Rhinotermitidae studied (Sillam-Dussès et al., 2009; Bordereau and Pasteels, 2011). The sternal gland of workers does not differ significantly in size from the sternal gland of females, but it differs in having a bilobed shape, as in workers of other rhinotermitids (Ampion and Quennedey, 1981). The estimated quantity of dodecatrienol in one gland (1–10 pg), and the estimated activity threshold (1 pg/cm), correspond to values observed in some *Reticulitermes* species (see Bordereau and Pasteels, 2011).

The activity threshold appears to be only slightly higher in females than in workers, as well as the content of dodecatrienol in the corresponding glands (Table 5). This is, once again, a similarity with *Reticulitermes*, i.e., *R. santonensis* (Laduguie et al., 1994a,b; Wobst et al., 1999). On the other hand, it contrasts with the dramatic differences between the amounts of dodecatrienol used by workers and females in the termitids *Pseudacanthotermes spiniger* (Bordereau et al., 1991, 1993) and *Cornitermes bequaerti* (Bordereau et al., 2002).

In summary, the chemistry of trail-following and sex attraction in *Psammotermes*, with dodecatrienol being used for both purposes, differs little from that of other subterranean rhinotermitids, despite the extremely different climatic conditions in which *Psammotermes* lives. Probably the most obvious adaptation to arid environment is the unprecedented speed of motion of both workers and imagos, probably to minimize the time spent in hot and arid areas during foraging and imaginal dispersal; their velocity is comparable to that of open-air foraging termites (pers. obs.). Interestingly, workers of *Psammotermes* do not easily follow their own sternal gland extracts. The orientation effect of the trail pheromone is weak in *Psammotermes*, while this is not the case in all other Rhinotermitidae studied, *Prorhinotermes* included. This weak orientation effect may be the due to the unprecedented speed of the individuals tested.

The surprising constancy of pheromonal signaling with ecological conditions has already been noted in distant taxa sharing the same pheromones, such as Kalotermitidae and Macrotermitinae (see Bordereau and Pasteels, 2011). The only singularity of *Psammotermes* within Rhinotermitidae is the production of the sex pheromone by both the sternal and the tergal glands of female imagos. On the other hand, the genus *Prorhinotermes*, supposedly a close relative of *Psammotermes* (Austin et al., 2004; Lo et al., 2004; Ohkuma et al., 2004; Inward et al., 2007), remains the sole genus of Rhinotermitidae with a diterpene (neocembrene) used as the major component of the trail pheromone, in addition to the minor component dodecatrienol (Sillam-Dussès et al., 2005, 2009).

Our findings underline the conservative nature of chemical communication in termites, with dodecatrienol being the most frequent component of pheromonal signals in trail-following and sex attraction in Rhinotermitidae and Termitidae. At the same time, they highlight the tight evolutionary relationship

between the trail following of working castes and the sex attraction of imagos, the ancestral life forms of termites; the former has supposedly developed from the latter during the social evolution of Isoptera (Traniello and Leuthold, 2000, Bordereau and Pasteels, 2011).

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Binding Characterization of Recombinant Odorant-binding Proteins from the Parasitic Wasp, *Microplitis mediator* (Hymenoptera: Braconidae)

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Abstract Chemoreception in insects is mediated by small odorant-binding proteins (OBPs) that are believed to carry lipophilic stimuli to the olfactory receptor cells through the aqueous sensillar lymph. Binding experiments and recent structural studies of OBPs have illustrated their versatility and ability to accommodate ligands of different shapes and chemical structures. We expressed and purified seven recombinant OBPs (MmedOBP1-MmedOBP7) from the parasitic wasp, *Microplitis mediator* (Hymenoptera: Braconidae) in a prokaryotic expression system. With 4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonic acid (bis-ANS) as a fluorescent probe, the ligand-binding specificities of these seven MmedOBPs with 50 small organic compounds were investigated *in vitro*. The results revealed that all of the *M. mediator* OBPs can bind a wide variety of odorant molecules with different binding affinities. The best ligand for all seven MmedOBPs was β-ionone. MmedOBP2 showed affinity for some aromatic compounds, whereas MmedOBP4 and MmedOBP6 bound several terpenoids. MmedOBP5 bound β-ionone, but did not bind any of the other potential ligands that we tested.

Key Words Binding specificity · 4,4′-Dianilino-1, 1′-binaphthyl-5,5′-sulfonate acid (bis-ANS) · Fluorescent probe · *Microplitis mediator* · Odorant-binding protein (OBP) · Polymerase chain reaction (PCR) · Protein expression · Hymenoptera · Braconidae

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Introduction

Microplitis mediator (Haliday) (Hymenoptera: Braconidae), is a solitary endoparasitoid. It is polyphagous on about 40 species of Lepidoptera, including an important pest of cotton in China, the bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Shenefelt, 1973). Generally, *M. mediator* searches and locates its host by relying on olfactory senses that detect specific odorants.

In insects, olfaction is mediated by odorant-binding proteins (OBPs), which are small antennal proteins that have been proposed to play an important role in perceiving and discriminating different odorants. OBPs are water-soluble acidic proteins with a pattern of six conserved cysteine residues. They are thought to carry lipophilic odorants to the olfactory receptor cells through the

hydrophilic environment of the sensillar lymph, and are involved in the initial biochemical step of odor reception (Vogt et al., 1985; Steinbrecht, 1996, 1999; Wang et al., 2003; Xu et al., 2005; Pelosi et al., 2006; Laughlin et al., 2008). There are many *in vitro* assays to explore the binding properties of OBPs to putative ligands. The first OBP was identified by using tritium-labeled pheromone (Vogt and Riddiford, 1981), and this isotope labeling method also was used to study the binding affinities among pheromones and pheromone-binding proteins (PBPs) (Du and Prestwich, 1995; Maida et al., 2000, 2003). However, fluorescent binding assays also have been conducted in many species, with 1-aminoanthracene (1-AMA),1-anilinonaphthalene-8-sulphonic acid (ANS), and N-phenyl-1-naphthylamine (1-NPN) as fluorescent probes (Campanacci et al., 2001; Bette et al., 2002; Calvello et al., 2003; Honson et al., 2003; Riviere et al., 2003). The following are representative examples with 1-NPN as the fluorescent probe. The OBP1 of the migratory locust, *Locusta migratoria* (Orthoptera: Acrididae) does not bind fecal or green leaf volatiles, but shows high affinity to some linear aliphatic compounds, with pentadecanol and 2-pentadecanone binding with the highest affinity (Jiang et al., 2009). The affinity of 12 compounds to three aphid OBPs were measured in competitive binding experiments. Similar behavior was observed for the three proteins towards most ligands, but (*E*)-β-farnesene, the alarm pheromone, and farnesol exhibited clear selectivity to OBP3 (Qiao et al., 2009). The silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae) PBP1, PBP2, GOBP2, and ABPx all bind the sex pheromone component (10*E*, 12*Z*)-hexadecadien-1-ol (bombykol). BmorPBP1, BmorPBP2, and BmorABPx also bind the pheromone component (10*E*,12*Z*)-hexadecadienal (bombykal) equally well, whereas BmorGOBP2 can discriminate between bombykol and bombykal (Zhou et al., 2009). The *Chilo suppressalis* GOBP2 has significant affinity to *cis*-11-hexadecenal (Z11-16: Ald), the main component of the sex pheromone and to laurinaldehyde and benzaldehyde, two general plant volatiles (Gong et al., 2009). By using a fluorescent displacement assay, Inkster et al. (2005) found that disparlure analogs all bound the gypsy moth, *Lymantria dispar* PBP1 with similar affinity to (−)-disparlure.

In previous research, we identified several putative OBPs in *M. mediator*, only four of which were found to be antennal specific. The remaining genes are expressed either ubiquitously or strictly regulated in specialized tissues or during different developmental stages. Some OBP genes are sex specific (Zhang et al., 2009). In order to further study the properties of these OBPs and their relationships with volatiles of host plants, we investigated the binding of 50 diverse small organic compounds to seven recombinant OBPs (MmedOBPs) from *M. mediator*. In these assays, 4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate acid (bis-ANS) was used as the fluorescent probe.

Methods and Materials

Insects A colony of *M. mediator* was obtained from the Institute of Plant Protection, Hebei Academy of Agriculture and Forestry, China. The parasitoids emerged from the host as mature larvae and spun silk cocoons for pupation. Emerged adult parasitoids were fed on a 10% honey solution and mated in culture cages (20×20×10 cm) at 26±1°C, 14L:10D (Wang et al., 1984). Antennae were dissected from

Table 1 Oligonucleotide primers used for expressing odorant-binding proteins for *Microplitis mediator*

Primer Name	Sequence (5′–3′)	Position (bp)
OBP1-forward	gtggatccAGAATGACTATGACACAGATTAG[a]	156–178
OBP1-reverse	gtgaattcTTATGGGAAGAAAACTATCTCTGGA	500–524
OBP2-forward	gtggatccAAATCGGTACAGAAACGAGAATG	131–153
OBP2-reverse	gtgaattcTTACGTGAACTTGTTATTGGTCTTG	478–502
OBP3-forward	gtggatccGATGATGACATGAAAGAAAAGCA	119–131
OBP3-reverse	gtgaattcTTACTCGTCATGGTGATGG	470–488
OBP4-forward	gtgaattcAATTCAAATATGGAGGAACTT	152–172
OBP4-reverse	gtgtcgacTTAATTATCAGATTTAGTGTTTATAG	498–523
OBP5-forward	gtggatccTTACAAGAGATAATGAACACATT	135–157
OBP5-reverse	gtgaattcTTATAAATGTTGGTTTTGATTT	523–544
OBP6-forward	gtggatccAGAATGAGTATGGCACAAAC	138–157
OBP6-reverse	gtgtcgacTTACGGGAAGAAGTATAACTCT	486–507
OBP7-forward	gtggatccAAGTTACCCGACTGGGTG	220–227
OBP7-reverse	gtgaattcTTAAATCATAAACCACATATCA	579–590

[a] Restriction enzyme sites for *Bam*HI(ggattc), *Eco*RI (gaatcc), and *Sal*I(gtcgac) are underlined

1- to 3-d-old females or males and immediately frozen in liquid nitrogen, and then stored at −70°C until use.

RNA Manipulation and cDNA Synthesis Total RNA was extracted by homogenizing antennae in Trizol™ reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, then reverse transcribed into single strand cDNA by using oligodT18 primers and MMLV reverse transcriptase (Promega, Madison,WI, USA). The reverse transcription reaction was performed at 42°C for 30 min, and the reaction was stopped by heating at 95°C for 5 min.

Recombinant Protein Expression and Purification The *M. mediator* OBPs (MmedOBP1–MmedOBP7) were obtained by prokaryotic expression in BL-21 *Escherichia coli* cells. Briefly, specific primers (Table 1) were used to amplify the coding sequence of MmedOBP genes with the cDNA as the template. The PCR product was first cloned into a pGEM-T simplevector (Takara, Dalian, Liaoning, China) and then excised and cloned into an expression vector pGEX-4T-1 for over-expression in prokaryotic cells BL21(DE3). The pGEX-4T-1 vector allows the expression of a recombinant product tagged with a glutathione *S*-transferase (GST) sequence at its N-terminus.

Clones containing the appropriate OBP sequence were chosen and incubated. When the optical density of the culture had reached a value of OD_{600}=0.6–0.8, protein expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. Cells were incubated for an additional 8 hr at 30°C; harvested by centrifugation at 5000×*g* for 15 min at 4°C; and cells were lysed by sonication (60×5 sec pulses of 400W) on ice. The recombinant OBPs both were present in the supernatant of the lysate and the pellet as inclusion bodies. Recombinant proteins in the supernatant were purified by GSTrap FF (GE Healthcare, Piscataway, NJ, USA). GST tags were cleaved from the recombinant proteins by thrombin on-column (GE Healthcare), then desalted by using HiTrap Desalting (GE Healthcare).

Fluorescence Binding Assay Emission fluorescence spectra were recorded on a fluorescence spectrometer (Lengguang F96, Shanghai, China) with a 1 cm light path quartz cuvette and 10 nm slits for both excitation and emission. The fluorescent probe 4,4′-dianilino-1,1′-binaphthyl-5, 5′-sulfonate (bis-ANS, Sigma, Riedstr, Steinheim, Germany) was excited at 365 nm and emission spectra were recorded from 435 to 580 nm. To measure the affinity of bis-ANS to each protein, a 2 μM solution of the protein in 50 mM Tris–HCl, pH 7.4, was titrated with aliquots of 1 μM of each potential ligand in methanol to final concentrations of 2–20 μM. The affinities of each OBP for 50 potential ligands (all purchased from Sigma-Aldrich, St. Louis, MO, USA, Supplementary Data Table 1) were measured in competitive binding assays by using both protein and bis-ANS at 2 μM in the presence of 1–50 μM of each competing ligand

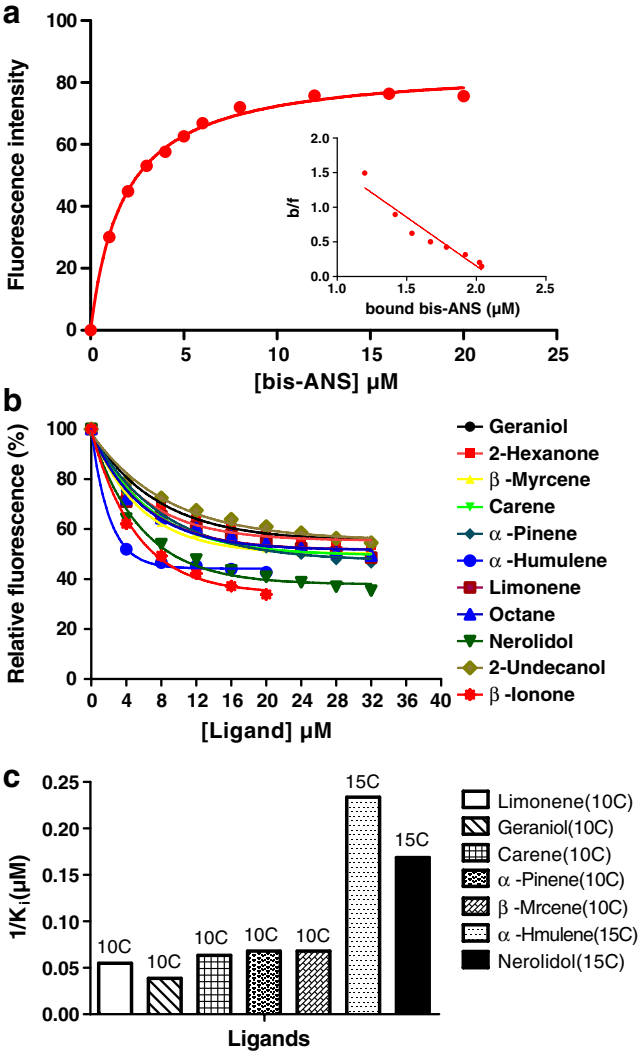


Fig. 1 Binding curve for 4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate acid (bis-ANS) to recombinant *Microplitis mediator* OBP4 (**a**) and Scatchard plot (inset). A 2 mM solution of the protein in Tris buffer was titrated with a 1 mM solution of bis-ANS in methanol to final concentrations of 2–20 μM. Binding curves also were measured for the other OBPS (data not shown) and dissociation constants (mean of three replicates) were: OBP1: 3.10±0.23 μM, OBP2: 3.70±0.37 μM, OBP3: 2.48±0.18 μM, OBP4: 1.99±0.14 μM, OBP5: 2.29±0.62 μM, OBP6: 5.63±1.02 μM, and OBP7: 3.54±0.16 μM. Competitive binding of selected ligands to *M. mediator* OBP4 (**b**). A mixture of the protein and bis-ANS in Tris, both at the concentration of 2 μM, was titrated with a 1 mM solution of each competing ligand to final concentrations of 2–32 μM. Fluorescence intensities are reported as percent of the values in the absence of competitor. The same potential ligands were also used with other OBPs (data not shown). Relationship between ligand size and K_i of terpenoids (**c**)

Data Analysis To determine the binding constants of bis-ANS to each OBP, the intensity values corresponding to the maximum of fluorescence emission were plotted against free ligand concentrations. Bound ligand was evaluated from the values of fluorescence intensity by assuming that the protein was 100% active, with a stoichiometry of 1:1 (protein:ligand) at saturation. The curves were linearized by using a Scatchard plot program (Campanacci et al., 2001). Dissociation constants of the competitors were calculated from the corresponding IC_{50} values, by using the equation: Ki = [IC50]/1 + [bis-ANS]/$K_{bis\text{-}ANS}$, where [bis-ANS] is the free concentration of bis-ANS and $K_{bis\text{-}ANS}$ is the dissociation constant of the protein/bis-ANS complex (Wei et al., 2008).

Results

Binding Specificity of MmedOBPs The seven purified proteins were used in competitive binding assays with the fluorescent probe bis-ANS as a reporter and the 50 potential ligands as competitors. First, we measured the dissociation constants between MmedOBPs and bis-ANS. Values ranged from 1.99 μM to 5.64 μM (Fig. 1a, b).

Competitive binding experiments revealed different specificities for the seven proteins (Table 2). MmedOBP1 bound 6 ligands, but only 2-Ethylhexanol (K_i=59.30 μM) and β-ionone (K_i=40.69 μM) effectively replaced bis-ANS from MmedOBP1. MmedOBP2 bound 7 ligands, whereas MmedOBP3-MmedOBP7 bound 3, 11, 1, 11, and 5

Table 2 Binding affinities of *Microplitis mediator* odorant-binding proteins for experimental ligands in a binding assay with the fluorescent probe 4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate acid (bis-ANS)

Potential ligand[a]	OBP1	OBP2	OBP3	OBP4	OBP5	OBP6	OBP7
2-Ethyl hexanol	59.30±2.73	N. D.[b]	N. D.	N. D.	N. D.	N. D.	N. D.
2-Hexanone	N. D.	N. D.	N. D.	25.14±0.74	N. D.	N. D.	N. D.
Acetophenone	N. D.	10.80±0.45	N. D.	N. D.	N. D.	N. D.	N. D.
3,4-Dimethylenzaldehyde	N. D.	3.40±0.11	N. D.	N. D.	N. D.	N. D.	N. D.
Benzaldehyde	N. D.	3.90±0.25	N. D.	N. D.	N. D.	N. D.	N. D.
4-Methylbenzaldehyde	N. D.	4.10±0.07	N. D.	N. D.	N. D.	N. D.	N. D.
Octanal	>100.00	N. D.	N. D.	N. D.	N. D.	65.83±1.90	N. D.
Octane	N. D.	N. D.	N. D.	17.96±0.57	N. D.	N. D.	N. D.
β-Ionone	40.69±1.21	10.60±0.59	51.28±0.73	4.37±0.07	>100.00	5.79±0.33	15.51±1.13
Isoamyl alcohol	>100.00	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
2-Heptanone	>100.00	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
2-Undecanol	N. D.	N. D.	N. D.	28.96±0.78	N. D.	20.13±0.22	N. D.
Pentane	N. D.	N. D.	N. D.	N. D.	N. D.	45.42±1.16	N. D.
Undecane	N. D.	N. D.	N. D.	N. D.	N. D.	69.87±2.53	N. D.
Nonyl acetate	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	>100.00
Citral	N. D.	4.30±0.05	>100.00	N. D.	N. D.	N. D.	N. D.
α-Pinene (10C)	N. D.	21.15±0.22	N. D.	14.72±0.65	N. D.	N. D.	N. D.
β-Pinene (10C)	N. D.	N. D.	N. D.	N. D.	N. D.	30.29±0.62	N. D.
β-Ocimene (10C)	N. D.	N. D.	>100.00	N. D.	N. D.	N. D.	N. D.
α-Humulene (15C)	N. D.	N. D.	N. D.	4.28±0.35	N. D.	22.97±1.30	N. D.
β-Humulene (15C)	N. D.	N. D.	N. D.	N. D.	N. D.	16.06±0.64	N. D.
β-Myrcene (10C)	N. D.	N. D.	N. D.	14.69±0.55	N. D.	25.57±1.09	N. D.
Carene (10C)	N. D.	N. D.	N. D.	15.74±0.62	N. D.	30.49±1.16	>100.00
α-Terpinene (10C)	N. D.	N. D.	N. D.	N. D.	N. D.	26.50±0.76	N. D.
Nerolidol (15C)	>100.00	N. D.	N. D.	5.93±0.29	N. D.	N. D.	>100.00
Limonene (10C)	N. D.	N. D.	N. D.	18.23±0.57	N. D.	N. D.	N. D.
Geraniol (10C)	N. D.	N. D.	N. D.	25.89±0.74	N. D.	N. D.	>100.00

Affinities (mean ± SD) measured in μM

[a] More potential ligands were tested, but the remaining 23 potential ligands did not bind any of the OBPs. These compounds were 3-hexanone, *Z*-hex-3-en-1-ol, ethylheptanoate, decanal, *E*-2-*Z*-6-nonadienal, *Z*-3-hexenyl acetate, 2-nonanone, *Z*-3-nonen-1-ol, 2-octanone, nonanal, 2-octanol, ethylphenylacetate, benzoic acid, methyl salicylate, methyl phenylacetate, ethyllaurate, *S*-(−)-limonene, β-citronellol, nerol, β-*E,E*-farnesol, (−)-carveol, carvacrol, and indole

[b] N.D. is an abbreviation for not determined, which means that no significant binding was detected in the assay

ligands, respectively. All MmedOBPs bound β-ionone, as well as some aliphatic compounds. Otherwise, the *M. mediator* OBPs exhibited some degree of broad selectivity towards the ligands tested. Although all seven OBPs bound β-ionone, the binding affinities were different. MmedOBP4 had the highest binding affinity (4.37 μM), whereas MmedOBP5 had the lowest (>100 μM). Both MmedOBP4 and MmedOBP6 bound 11 ligands, but MmedOBP4 had a higher binding affinity than MmedOBP6.

Discussion

We explored the ligand binding specificity of seven MmedOBPs to 50 compounds including green leaf volatiles, aliphatic alcohols, aromatic compounds, terpenoids, and heterocyclic compounds. *Microplitis mediator* is polyphagous on hosts that include important agricultural pests for maize, cotton, and tobacco, such as *Helicoverpa armigera*, *Helicoverpa assulta*, *Heliothis virescens*, and *Spodoptera exigua* (Shenefelt, 1973). Successful parasitization requires a series of successive steps. These include host habitat location, host location, host acceptance, host suitability and host regulation. In the first three steps, volatiles have been reported to play important roles (Vet and Dicke, 1992). Such compounds may come directly from the host, as well as from host by-products, host food plants, host damaged food plants, or organisms closely associated with the host (Tumlinson et al., 1992; Steiner et al., 2007).

Of the 50 compounds examined, 14 were from cotton, healthy or damaged by *H. armigera* (Yu et al., 2007); ten were from maize, healthy or damaged by *Spodoptera frugiperda*, *S. exigua*, *H. virescens*, or *H. zea* (Alborn et al., 1997; De Moraes et al., 1998; Carroll et al., 2006); and seven were from tobacco, healthy or damaged by *H. armigera*, *H. assulta*, *H. virescens*, or *H. zea* (De Moraes et al., 1998; Yan et al., 2005). Therefore, most compounds used in our experiments were typical volatiles from the host plants. In this study, we used bis-ANS as a fluorescent probe, and bis-ANS emits a strong fluorescence in hydrophobic environments, but almost no fluorescence in water (Rosen and Weber, 1969). There is a central hydrophobic cavity in the conserved domain of the OBPs that can bind and transport hydrophobic molecules, including odorants. We showed that bis-ANS binds to all seven MmedOBPs, and emits fluorescence strongly. This suggests that bis-ANS may bind in the hydrophobic cavity of MmedOBPs.

de Bruyne and Baker (2008) reported that OBPs can be selected to detect key odorants with high sensitivity and to perceive a wide variety of general odorants at higher doses. Nonanal, decanal, and (*Z*)-3-hexenyl acetate elicited electroantennogram (EAG) responses from *M. mediator* (Yu et al., 2010), but they did not bind to all seven MmedOBPs in the test. This suggests that there may be other OBPs in *M. mediator* antennae. In contrast, octanal bound to MmedOBP1 and MmedOBP6 in our experiment, but it did not elicit behavioral responses from *M. mediator* in a previous study (Yu et al., 2010).

MmedOBP1, MmedOBP3, and MmedOBP7 showed weaker binding ability to all ligands, whereas MmedOBP5 only bound β-ionone. Similarly, in *Apis mellifera*, general odorant-binding protein (GOBP) ASP2 has the ability to bind ligands of different structures and properties (Lescop et al., 2009), and these authors propose that the broad ligand specificity of GOBPs compared with PBPs is conferred by the cumulative effects of weak nonspecific protein–ligand interactions and of enhanced protein internal dynamics at the ligand entry site.

We presumed that MmedOBPs can recognize the chemical conformation and functional groups of ligands. MmedOBP1, MmedOBP2, MmedOBP3, MmedOBP4 and MmedOBP7 can distinguish citral and nerolidol, respectively. Three MmedOBPs (MmedOBP2, MmedOBP4, and MmedOBP6) can bind to terpenoids effectively. MmedOBP4 mainly expressed in female antennae, about 99.6% in female antennae and 0.4% in male antennae. MmedOBP6 was expressed almost equally in female and male antennae. The expression level of MmedOBP2 was higher in male antennae than in female antennae (Zhang et al., 2009). Due to the function of terpenoids in parasitoid finding hosts, we concluded that those proteins may play important roles for *M. mediator* to search and locate the hosts. When MmedOBP4 and MmedOBP6 bind to terpenoids, they have stronger binding affinity to C15 terpenoids than C10 terpenoids (Fig. 1c).

In this study, we successfully expressed seven MmedOBPs in *E. coli* and purified them by affinity chromatography. We further characterized the ligand-binding specificities of those OBPs to 50 diverse small organic compounds by using bis-ANS as probe. The results could help us to understand the mechanism of olfactory recognition when parasitoids search for and locate their hosts.

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Glucose and Glucose Esters in the Larval Secretion of *Chrysomela Lapponica*; Selectivity of the Glucoside Import System from Host Plant Leaves

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Abstract Larvae of *Chrysomela lapponica* (Coleoptera: Chrysomelidae) sequester characteristic *O*-glucosides from the leaves of their food plants, namely *Betula* and/or *Salix* The present study focuses on birch-feeding larvae of *C. lapponica* from the Altai region in East Kazakhstan. As in other sequestering leaf beetle larvae, the compounds are transported intact *via* different membrane barriers into the defensive system, followed by glucoside cleavage and subsequent transformations of the plant-derived aglycones. Unlike previous studies with model compounds, we studied the sequestration of phytogenic precursors by analyzing the complex pattern of glucosides present in food plant *Betula rotundifolia* (39 compounds) and compared this composition with the aglycones present as butyrate esters in the defensive secretion. In addition to the analytic approach, the insect's ability, to transport individual glucosides was tested by using hydrolysis-resistant thioglucoside analogs, applied onto the leaf surface. The test compounds reach the defensive system intact and without intermediate transformation. No significant difference of the transport capacity and selectivity was observed between larvae of birch-feeding population from Kazakhstan, and previous results for larvae of birch-feeding population from the Czech Republic or willow-feeding populations. Overall, the transport of the phytogenic glucosides is highly selective and highly efficient, since only minor compounds of the spectrum of phytogenic glucoside precursors contribute to the limited number of aglycones utilized in the defensive secretion. Interestingly, salicortin **44** and tremulacin **60** were found in the leaves, but no aldehyde or esters of salicylalcohol. Surprisingly, we observed large amounts of free glucose, together with small amounts of 6-*O*-butyrate esters of glucose (**27a/b** and **28a/b**).

Key Words *O*-glucosides · Butyrate esters · Salicortin · Tremulacin · Salicylaldehyde · *Betula* · *Salix* · Coleoptera · Chysomelidae · Antipredatory · Beetle defense

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Introduction

Larvae of the leaf beetles (Chrysomelina) possess nine pairs of dorsal exocrine glands from which secretions emerge as small droplets when a predator appears. As soon as the disturbance is over, the droplets are resorbed into a large reservoir into which glandular cells open. The anti-predatory effect of the secretion is based either on *de novo* production of repellents or on compounds derived from sequestered, plant-derived glucosides. The major components, which are secreted by leaf beetle larvae belonging to the taxa *Phaedon*, *Gastrophysa*, *Hydrothassa*, *Prasocuris Plagiodera*, *Linaeidea*, and *Phratora* (in part) are iridoid

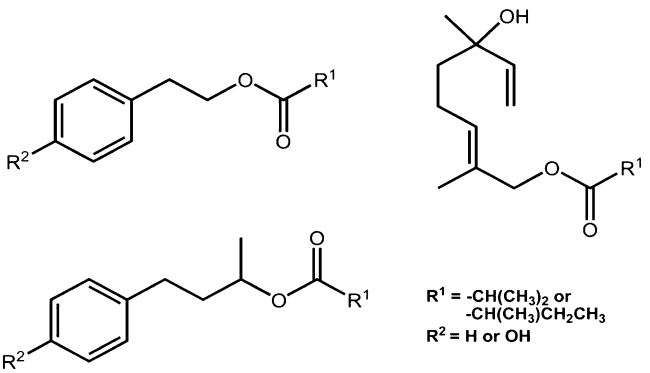
monoterpenes (Meinwald et al. 1977; Blum et al. 1978; Pasteels et al. 1982; Soe et al. 2004). According to (Termonia et al. 2001) the *de novo* production of iridoids is considered the ancestral strategy. Recent studies have demonstrated, however, that the whole group also is able to sequester early precursors from plants (Feld et al. 2001; Kuhn et al. 2004; Kunert et al. 2008). A more advanced and resource saving strategy to produce chemical defenses, is the sequestration of plant-derived precursors. The sequestration of phenol glucosides, especially salicin, has been developed and optimized by species feeding on Salicaceae, i.e., *Phratora vitellinae* and *Chrysomela* (Pavan 1953; Pasteels et al. 1983). In these species, the plant-derived glucosides serve as precursors for the repellent salicylaldehyde; a concomitant *de novo* biosynthesis does not seem to play a significant role.

The most derived Chrysomelina species, the interrupta group *sensu* (Termonia and Pasteels 1999), which includes *Chrysomela lapponica* do not sequester single glucosides, but import a broad spectrum of glucosidically bound plant-derived alcohols (Kuhn et al. 2007). After import into the reservoir, which functions as a bioreactor containing all necessary enzymes for the transformation of the sequestered precursors, the glucose moiety is removed, and the aglycones are esterified with isobutyric acid, 2-methylbutyric acid, and benzoic acid generated from the insects' internal pools of amino acids. This type of "combinatorial biosynthesis" can generate a cocktail of more than 70 secretion ingredients, as has been shown for a larval secretion of *C. lapponica* from Bavaria, Queyras, Czech Republic, and related North American species (Hilker and Schulz 1994; Schulz et al. 1997; Termonia and Pasteels 1999; Kuhn et al. 2007). Bioassays have demonstrated the protective function of these blends against predators (Hilker and Schulz 1994). For several defensive secretions of leaf beetle larvae, antimicrobial effects also have been demonstrated and discussed (Gross et al. 2002).

The sequestration of glucosides as pro-toxins is advantageous. The compounds often occur in high abundancy in the food plants. The polar glycosides are not able to pass membranes unless functional transport systems mediate their passage, which assures safe guidance of the compounds to their destination through the larval body. The leaf beetle larvae possess an interconnected network of transport systems for uptake and excretion of surplus glucosides (Discher et al. 2009).

According to model experiments with selected (thio) glucoside mixtures, the phytogenic precursors are imported via the gut membrane with low selectivity, followed by a specific import of only a few compounds from the hemolymph into the reservoir. Model studies were based on test compounds, comprising a glucoside precursor of iridoid biosynthesis, salicin for salicylaldehyde production, and phenylethanolglucoside that is converted into the corresponding (iso)butyrate (Scheme 1). Uptake into the glandular reservoir was highly specific, and only the phytogenic precursors for the genuine defenses accumulated in the reservoir. For example, only salicin was imported into the reservoir of *Chrysomela populi* and converted to salicylaldehyde. In contrast, the iridoid-producing larvae of *Phaedon cochleariae* accumulated only the glucoside of 8-hydroxy-geraniol, which is converted into the iridoid chrysomelidial (Discher et al. 2009). This is different in the case of the defensive secretion of *C. lapponica* feeding on birch. Their defensive secretion is a rather complex mixture composed of butyrate esters of very different and most likely plant-derived aglycones released from sequestered glucosides (Hilker and Schulz 1994; Schulz et al. 1997; Kuhn et al. 2007). This implies that larvae of *C. lapponica* possess either several different transport systems, each of them specifically tuned to the imported glucoside, or that the larvae possess a transport system of low selectivity that allows the import of a broad range of glucosides, as demonstrated by Kuhn et al. 2007 with *C. lapponica* from Queyras feeding on willow by using thioglucosides as probes.

In this study, we focused on birch feeding *C. lapponica* from Kazakhstan, which also produce butyrate esters, but lack the production of salicylaldehyde, as will be shown. To investigate the capacity and the "filter characteristics" of the transport systems that operate in larvae of birch feeding *C. lapponica*, we compared the spectrum of the glucosides present in the leaves of the host plant *Betula rotundifolia* with the spectrum of butyrate esters in the defensive secretion of the larvae. Here, we demonstrated that the defensive secretions and the glucoside spectrum of *B. rotundifolia* share only a small number of aglycones that are sequestered and utilized by the insect. Moreover, the defensive secretion of *C. lapponica* contains large amounts of glucose and certain glucose esters (butyrates), which may serve as cryoprotection.



Scheme 1 Butyrate esters from the defensive secretion of larvae of birch-feeding larvae of *Chrysomela lapponica* endemic to the Altai region, East Kazakhstan

Methods and Materials

Leaf Beetle Larvae and Host Plant Leaves Larvae of *C. lapponica* feeding on leaves of *Betula rotundifolia* were collected in the Altai mountains (East Kazakhstan) near the Burkhat Pass (2130 m altitude, 49°07,438'N, 86°01,365'E) in early August 2008. Larvae were reared for 1 week with leaves from *Betula rotundifolia* from the Burkhat Pass. Freshly harvested *Betula* twigs were kept alive for several days in tap water. Additional plant material was sun dried and stored for phytochemical analysis. Field conditions did not allow for extraction of fresh plant material.

Collection of Defensive Secretion Larvae were gently squeezed by forceps and forced to protrude their mesothoracal glands. The emerging droplets were collected with glass capillaries (Hirschmann Laborgeräte, diam: 0.28 mm, length: 100 mm). The capillaries were sealed by melting with a micro gas torch and stored at −20°C.

Analytical Procedures Analysis of defensive secretions. An aliquot of the secretion (1-3 μg) was dissolved in 20 μl dichloromethane. After vortexing and centrifuging, 1 μl was subjected to GC-EIMS analysis (ThermoQuest Finnigan Trace GC-MS 2000 (Quadrupole) equipped with Alltech EC 5-column, 15 m×0.25 mm, film thickness 0.25 μm). Substances were separated using helium as carrier (1 ml/min); conditions: 45°C (2 min), then at 10°C min^{-1} to 280°C (5 min).

Highly concentrated samples were appropriately diluted. For quantification, external standards at different concentrations were used. Polar components were analyzed after derivatization with MSTFA. The defensive secretion (5 μl solution in dichloromethane) was mixed with *N*-methyltrimethylsilyltrifluoroacetamide (MSTFA) (10 μl) and pyridine (5 μl). After heating to 70°C for 1 h, an aliquot (1 μl) of the solution was analyzed directly by GC-EIMS. Free fatty acids were derivatized with pentafluorobenzylbromide (PFBB) prior to analysis (Attygalle et al. 1991). For esterification, the secretion (0.5 μl) was dissolved in triethylamine (10 μl) containing PFBB (5 μl). The solution was kept at 25°C for 3 h and hydrolyzed with water (30 μl). After extraction with *n*-hexane (20 μl), the solution was analyzed by GC-EIMS (conditions see above). All compounds were identified by comparison with authentic references and sample chromatograms, respectively. Secretion analysis was carried out against external standards (N=3). No replicates were measured owing to the restricted availability of secretion.

Transport Analysis with Thioglucosides Leaves from *Betula pendula* (used instead of *B. rotundifolia* because of the larger surface area for the transport experiments) were painted on the upper side with 0.5 ml of MeOH/H_2O (1:1, v/v) containing the test compound at a concentration of 25 mM. After evaporation of the solvent with an air stream, third instars (four larvae per ca. 3.5 cm^{-2} leaf segment) were allowed to feed on the treated leaves for 48 h, followed by sampling of defensive secretion. The defensive secretion was withdrawn with a small capillary from the everted glands of the larvae. The secretion was stored and transported in sealed capillaries. The thioglucosides transported into the glandular system were analyzed and quantified as described (Kuhn et al. 2007).

Statistics To obtain valid data, feeding experiments with thioglucosides were repeated several times. *Chrysomela lapponica* (Kazakhstan): compounds **1** to **6** (N=6), *C. lapponica* (Czech Republic): compound **1** (N=5), compounds **2**, **4**, and **6** (N=4). Variances between groups were not homogeneous according to Levene's test. Hence, Mann-Whitney test was used to evaluate significant differences. Statistics were calculated with SPSS Statistics 17.0.

Analysis of Leaf Glycosides Air dried leaves (3 g) of *B. rotundifolia* were pestled in liquid nitrogen and extracted with 20 ml 80% aqueous methanol using an Ultra-Turrax homogenizer for 3 min. Extraction was continued by shaking for 1 h at ambient temperature. Solids were removed by filtration and re-extracted with methanol (20 ml) for another 1 h at ambient temperature. The combined extracts were evaporated to dryness, re-dissolved in methanol (10 mg/ml), and after filtration (0.45 μm pore size) the glycosides were determined by HPLC using a Thermoquest LCQ (Thermoquest, D-63329 Egelsbach, Germany) in the APCI mode (vaporizer temperature: 560°C) connected to an Agilent HP1100 HPLC-system equipped with an RP18 column, (Purosphere 4×250 mm (5 μm). Samples (10 μl) were analyzed by using gradient elution at 0.65 ml min^{-1}. Solvent A: 0.5% acetic acid in water; solvent B: 0.5% acetic acid in acetonitrile. Compounds were eluted according to the following protocol: 0-10 min, 10% of B in A; 10–50 min 10–60% of B in A. UV-detection at 254, 266, and 360 nm prior to the mass spectrometer allowed us to gain further information on the analytes. Commercially unavailable compounds were isolated and identified based on data from HR-ESI-MS, ^{1}H- and HSQC-NMR spectroscopy, and literature data. Known compounds were compared by their retention times and UV-Vis and MS data with authentic references. Myricetin derivatives were identified after hydrolysis of the crude extract of *B. rotundifolia* (1.2 M HCl for 4 h at 100°C) by HPLC using an authentic reference. In addition, the aglycones were determined after enzymatic hydrolysis (Schulz et al. 1997) of the raw extracts followed by GC-EIMS. Compounds were identified using authentic standards.

Table 1 Identification and quantification of defensive substances in the birch-feeding population of *Chrysomela lapponica* from the Burkhat Pass, East Kazakhstan

Peak number	Substance	Compounds [nmol/mg secretion]
	Acetic acid	3.6[a]
	Isobutyric acid	1.1[a]
	2-Methylbutyric acid	0.3[a]
1	Benzyl alcohol	0.3
2	(*Z*)-3-Hexenylisobutyrate	2.2
3	Hexylisobutyrate	1.2
4	5-Hexenyl isobutyrate	Not quantified[b]
5	Benzoic acid	trace
6	(*Z*)-3-Hexenyl-2-methylbutyrate	5.3
7	Hexyl-2-methylbutyrate	1.5
8	5-Hexenyl-2-methylbutyrate	Not quantified[b]
9	Benzylisobutyrate	8.7
10	Benzyl-2-methylbutyrate	4.6
11	Phenylethylisobutyrate	1.5
12	Phenylethyl-2-methylbutyrate	1.8
13	1,3-Hexandiyl-1,3-diisobutyrate	Not quantified[b]
14	(*Z*)-3-Hexenylbenzoate	trace
15	8-OH-Geranylisobutyrate	Not quantified[b]
16	8-OH-Linalylisobutyrate	0.1
17	mixture of 1,3-Hexandiyl-1-isobutyrate-3-(2-methylbutyrate), 1,3-Hexandiyl-1-(2-methylbutyrate)-3-isobutyrate	Not quantified[b]
18	8-OH-Geranyl-2-methylbutyrate	Not quantified[b]
19	*p*-OH-Phenylethylisobutyrate	13.0
20	8-OH-Linalyl-2-methylbutyrate	0.2
21	1,3-Hexandiyl-1,3-di-2-methylbutyrate	Not quantified[b]
22	*p*-OH-Phenylethyl-2-methylbutyrate	25.0
23	Betuligenolisobutyrate	4.2
24	Betuligenol-2-methylbutyrate	4.3
25	Linolenic acid methylester	1.6
26a–e	Glucose	468.7
27a,b	Glucose-6-*O*-isobutyrate	17.2
28a,b	Glucose-6-*O*-2-methylbutyrate	26.8

Quantification was carried out using external standards (N=3), [a] quantified after derivatization with PFBB (Attygalle et al. 1991), [b] determined by spectral data comparison. Numbering in Table 1 corresponds peak numbering of Fig. 1.

Synthesis of Glucose Esters Acylation was carried out according to a modified method from Woudenberg (Woudenberg-van Oosterom et al. 1995). The procedure was conducted under an inert atmosphere. 100 mg glucose were dissolved in 10 ml *t*-butanol. 1 g molecular sieve 4 Å was added. 10 ml 12.5% ethylisobutyrate and ethyl-2-CH_3-butyrate, respectively, in *t*-butanol then were added. The reaction was started by adding 100 mg *Candida antarctica* lipase B. The suspension was stirred for 72 h at 50°C. The molecular sieve and the immobilized enzyme were separated by filtration, and the solvent was evaporated. The raw product was fractionated by column chromatography ($CHCl_3$/MeOH/H_2O=75/22/3) to yield the acylated glucoses. The described procedure produced an anomeric mixture. Hence, multiple signals were observed. Assignment of peaks was carried out using 2D-NMR-experiments (HSQC and HMBC).

Spectral Data of 6-O-isobutyryl-a/b-D-glucopyranoside ^{1}H NMR ($MeOD_4$) [ppm]: 5.01 (d, J=3.67 Hz, 0.5 H), 4.41 (d, J=7.74 Hz, 0.5 H), 4.30 (ddd, J=19.13 Hz, J=11.88 Hz, J=2.15 Hz, 1 H), 4.15-4.08 (m, 1 H), 3.89 (ddd, J=10.17 Hz, J=5.48 Hz, J=2.17 Hz, 0.5 H), 3.60 (t, J=9.29 Hz, 0.5 H), 3.43-3.38 (m, 1 H), 3.30-3.20 (m, 1 H), 3.17 (masked by

$MeOD_4$ signal, 1 H), 3.08-3.04 (t, J=8.69 Hz, 0.5 H), 2.51 (sept, J=7.00 Hz, 1 H), 1.10 (d, J=7.01 Hz, 6 H). ^{13}C NMR ($MeOD_4$) δ [ppm]: 178.8/178.7, 98.3/94.0, 78.0/73.8, 76.2/74.8, 75.4/70.8, 72.0/71.7, 64.9/64.8, 35.2/35.2, 19.4/19.3. MS (70 eV) m/z (%): 233(0.5), 219(3), 203(3), 173(5), 161 (5), 143(5), 131(18), 103(17), 89(28), 71(100), 60(19), 43 (97). ESI-HR-MS m/z $[M+H]^+$: calculated for $C_{10}H_{19}O_7$: 251.25366; found: 251.11276.

Spectral Data for 6-O-(2-methylpropanyl)-α/β-D-glucopyranoside ^1H NMR ($MeOD_4$) [ppm]: 5.03 (d, J= 3.53, 0.5 H), 4.42 (d, J=7.83, 0.5 H), 4.38-4.30 (m, 1 H), 4.16-4.09 (m, 0.5 H), 3.90 (dd, J=10.04 Hz, J=5.02 Hz, 0.5 H), 3.62 (t, J=9.42 Hz, 0.5 H), 3.44-3.39 (m, 0.5 H), 3.32-3.26 (m, 1 H), 3.24 (masked by $MeOD_4$ signal, 1 H), 3.08 (dd, J=9.62 Hz, J=7.90 Hz, 0.5 H), 2.40-2.30 (m, 1 H), 1.67-1.56 (m, 1 H), 1.48-1.38 (m, 1 H), 1.09 (td, J= 6.91 Hz, J=0.92 Hz, 3 H), 0.86 (dt, J=7.47 Hz, J=1.03 Hz, 3 H). ^{13}C NMR ($MeOD_4$) δ [ppm]: 178.4/178.3, 98.2/94.0, 78.0/73.8, 76.2/74.8, 75.5/70.8, 72.0/71.8, 64.8/64.7, 42.5/42.3, 28.0/27.9, 17.1/17.0, 12.0/11.9. MS (70 eV) m/z (%): 233(2), 217(2), 187(4), 175(4), 145(10), 103(27), 85(66), 74(37), 57(100). ESI-HR-MS m/z $[M+Na]^+$: calculated for $C_{11}H_{20}O_7Na$: 287.26207; found 287.11025.

Spectral Data of Santin-7-O-glucoside yellow amorphous powder; UV λ_{max} nm: 272, 338 (MeOH); HR ESI-FTMS *m/z* 507.14884 (($[M+H]^+$, calcd. for $C_{24}H_{27}O_{12}$, 507.14970), ^1H NMR (DMSO-d_6) δ: 12.61 (s, 1 H), 8.04 (d, J=8,8 Hz, 2 H), 714 (d, J=8.8 Hz, 2 H), 6.99 (s, 1 H), 5.12 (d, J=7.1 Hz, 1 H), 3.86 (s, 3 H), 3.79 (s, 3 H), 3.18-3.75 (sugar H), ^{13}C NMR (DMSO-d_6) δ:178.4, 161.5, 156.6, 155.9, 152.1, 151.4, 137.9, 132.3, 130.1, 122.2, 114.4, 106.3, 100.1, 94.2, 77.3, 76.7, 73.2, 69.5, 60.6, 60.4, 59.8, 55.6.

Results

The defensive secretion of the larvae of *C. lapponica* mainly comprised 2-methylbutyric and isobutyric acid esters of aglycones, which occur as glucosides in the leaves of the host plant (Table 1). Major compounds are the butyrate esters of hexanol (**3** and **7**), (*Z*)-3-hexenol (**2** and **6**), benzyl alcohol (**9** and **10**), phenylethanol (**11** and **12**), *p*-OH-phenylethanol (**19** and **22**), and betuligenol (**23** and **24**). While the concentration of free alcohols in the secretion was low, significant amounts of free 2-methylbutyric- and isobutyric acids along with acetic acid and traces of benzoic acid were found after derivatization with pentafluorobenzylbromide (Table 1). In addition, the secretion contained large amounts of free glucose (**26a-e**) along with glucose-6-*O*-butyrates (**27a/b** and **28a/b**), which are identified for the first time in leaf beetle defenses. Glucose and the two 6-*O*-glucose esters **27a/b** and **28a/b** were identified after derivatization with MSTFA by GC-EIMS using synthetic references. Identified compounds are compiled in Table 1 according to the elution order shown in Fig. 1.

The presence of large amounts of glucose was unexpected. Even more remarkable was the presence of C(6) glucose esters, indicating that the easily accessible primary hydroxy group of glucose can serve as a substrate for the acyl transferase(s) that catalyze the acylation of the plant-derived aglucones. Only the 6-*O*-butyrates **27a/b** and **28a/b** (a and b refer to α- and β-anomers of the butyrates) were detected (Scheme 2). Esters linked to a secondary hydroxyl group of the glucose moiety were not observed.

Glycosides in Leaves of B. rotundifolia We addressed the full spectrum of glycosides from dry leaves of the food

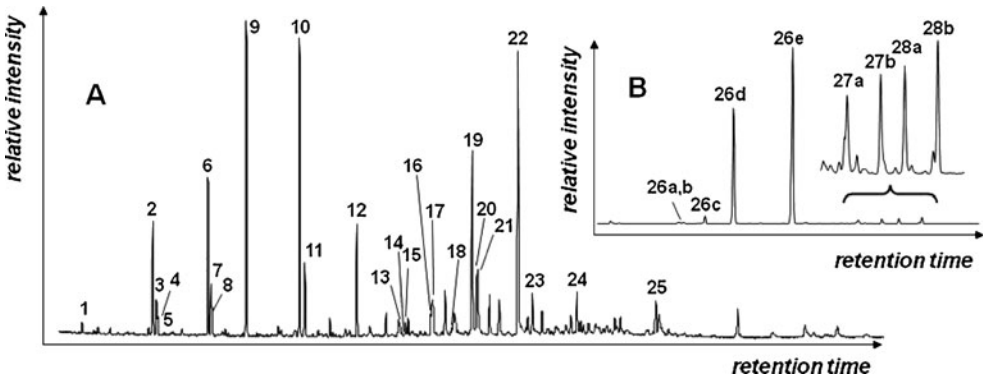
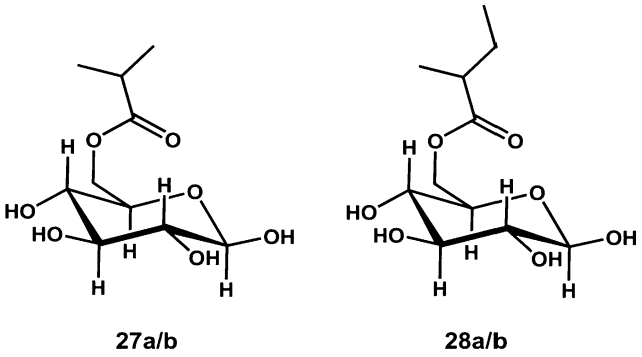


Fig. 1 Gaschromatographic profile of the defensive secretion of birch-feeding larvae of *Chrysomela lapponica* from the Altai Mountains, East Kazakhstan. Numbering and identification of the peaks corresponds to Table 1. **a** GC-EIMS analysis of the volatile esters of the secretion **b** GC-EIMS analysis of the polar constituents of the defensive secretion after derivatization with MSTFA. Low boiling silyl-derivatives are not shown. The four signals for **27a/b** and **28a/b** correspond to the α- and β-enantiomers of the silylated glucose esters. Silylated glucose is represented by the signals **26a-e**



Scheme 2 Butyrate esters (**27a/b**) and (**28a/b**) of glucose

plant *B. rotundifolia* by exhaustive extraction and analysis by HPLC-MS, LC-UV, and NMR of isolated compounds. The LC-profile (Fig. 2) of the polar extract of *B. rotundifolia* displayed a rich pattern of glycosylated flavonoids (**45**), (**46**), (**49**), (**53**) derived from quercetin (**58**), kaempferol (**62**), and the 3′,5′-trihydroxy-6,7,4′-trimethoxyflavones (**55**), and (**66**) (Table 2). To our knowledge, santin-7-*O*-glucoside (**59**) is isolated for the first time. The presence of tremulacin (**60**) and salicortin (**44**), which can ultimately serve for the production of salicylaldehyde after hydrolysis in the gut (Julkunen-Tiitto and Meier 1992), is noteworthy, since no butyrate esters of saligenin or free salicylaldehyde were found in the larval secretion. In general, comparison of the host plant chemistry with secretion revealed that the glycoside pattern of the host plant dramatically differed from the aglycone pattern found in the larval secretion. The predominant flavonoid glycosides were completely absent in the larval secretion, while the low molecular weight aromatic/phenolic esters and aliphatic hexenyl esters, which are minor or even trace components of the plant-extracted glycosides, dominated the larval secretions. Nevertheless, the aglycones of several plant-produced precursors like betuloside (**37**), phenylethyl-Glc (**42**), salidroside (**29**), and 8-OH-linaloyl-Glc (**39**) matched the aglycones present in the secretion. The identities of the 39 identified compounds are given in Table 2.

Since not all aglycones present in the defensive secretion were found as glucosides, we hydrolyzed the total extract with a mixture of glucosidases and galactosidases according to (Schulz et al. 1997). Analysis of the released compounds indeed revealed the presence of (*Z*)-3-hexenol, and benzyl-alcohol, which for unknown reasons were not found among the leaf-derived glycosides.

Hemolymph Transport of the Phytogenic Glycosides After uptake into the digestive system, the gut epithelial cells constitute the first membranes that the glycosides have to pass. Previously, we tested this passage in birch-feeding larvae from the Czech Republic and willow-feeding larvae of *C. lapponica* from Queyras by using hydrolysis-resistant thioglucosides (Kuhn et al. 2007). After application of the compounds to the leaf surface, larvae of the birch-feeding *C. lapponica* were allowed to feed on the treated leaves, and the amount of thioglucosides transported through the insect into the glandular reservoir was determined by HPLC-MS.

As shown (Fig. 3), the birch-feeding populations of *C. lapponica* from Kazakhstan and also from the Czech Republic (Kuhn 2005) show similar transport characteristics. No significant differences could be evaluated for thiosalicin (**1**), *o*-kresolthioglucoside (**2**), or 8-OH-geraniolthioglucoside (**6**). Only the uptake of phenylethylthioglucoside (**4**) differed significantly between the birch-feeding populations. Common to both population (birch and willow feeders) (Kuhn et al. 2007), is the selective import of only thioglucosides; galactosides were not taken up. Moreover, the birch-feeding

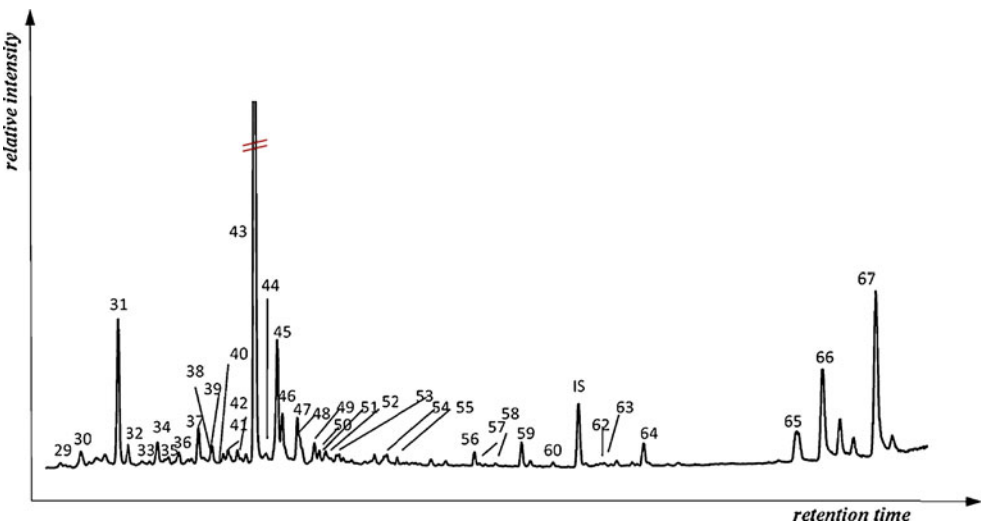


Fig. 2 HPLC-MS chromatogram of the methanolic extract of *Betula rotundifolia*. Compounds of the methanolic extract were separated and monitored by HPLC-MS on Purosphere RP18, 5 μm, 4×250 mm (Merck) using an isocratic solvent system 10% of B (10 min) and linear gradient from 10% B to 60% B (50 min). Solvent A: 0.5% CH_3COOH in H_2O, solvent B: 0.5% CH_3COOH in CH_3CN. Flow rate 0.65 ml/min. Numbering and identification of the peaks correspond to Table 2

Table 2 Identification and quantification of glucosides and other compounds from the polar extract of *Betula rotundifolia*

Peak	Molecular formula	$[M+H]^+$ *m/z*	Compound
29	$C_{14}H_{20}O_7$	301	Salidroside[a]
30	$C_{20}H_{22}O_{10}$	423	Catechin-7-0-xylopyranoside[d]
31	$C_{16}H_{18}O_9$	355	Chlorogenic acid [a]
32	$C_{15}H_{14}O_6$	291	(+)-Catechin [a]
33	$C_{19}H_{32}O_8$	389	Blumenol B – 9-O-glucoside[a]
34	$C_{19}H_{30}O_8$	387	Blumenol derivative[d]
35	$C_{16}H_{18}O_9$	355	Neochlorogenic acid[d]
36	$C_{15}H_{14}O_6$	291	(-)-Epicatechin[a]
37	$C_{16}H_{24}O_7$	329	Betuloside[b], (Smite et al. 1993)
38	$C_{27}H_{30}O_{17}$	627	Myricetin-rutinoside[c]
39	$C_{16}H_{28}O_7$	333	8-Hydroxylinaloyl-glucoside[d]
40	$C_{21}H_{20}O_{13}$	481	Myricetin-glucoside[c]
41	$C_{21}H_{20}O_{13}$	481	Myricetin-galactoside[c]
42	$C_{14}H_{20}O_6$	285	Phenethyl-glucoside[a]
43	$C_{27}H_{30}O_{16}$	611	Quercetin-3-O-rutinoside[a]
44	$C_{20}H_{24}O_{10}$	425	Salicortin[a]
45	$C_{21}H_{20}O_{12}$	465	Quercetin-3-O-glucoside[a]
46	$C_{21}H_{20}O_{12}$	465	Quercetin-3-O-galactoside[a]
47	$C_{27}H_{30}O_{15}$	595	Kaempferol-3-O-rutinoside[a]
48	$C_{28}H_{32}O_{16}$	625	Isorhamnetin-3-O-rutinoside[a]
49	$C_{20}H_{18}O_{11}$	435	Quercetin-3-O-arabinoside[b], (Hansen et al. 1999)
50	$C_{19}H_{32}O_7$	373	Blumenol C – 9-O-glucoside[a]
51	$C_{21}H_{20}O_{11}$	449	Kaempferol-3-O-glucoside[a]
52	$C_{22}H_{22}O_{12}$	479	Isorhamnetin-3-O-glucoside[a]
53	$C_{21}H_{20}O_{11}$	449	Quercetin-3-O-rhamnoside[a]
54	$C_{21}H_{20}O_{10}$	433	Kaempferol-3-O-rhamnoside[b], (Chung et al. 2004)
55	$C_{24}H_{26}O_{13}$	523	5.3′.5′-Trihydroxy-6.7.4′-trimethoxyflavone+hexose[d]
56	$C_{23}H_{24}O_{11}$	477	Pectolinaringenin-7-O-glucoside[b], (Yim et al. 2003)
57	$C_{22}H_{22}O_{10}$	447	Acacetin+hexose[d]
58	$C_{15}H_{10}O_7$	303	Quercetin[a]
59	$C_{24}H_{26}O_{12}$	507	Santin-7-O-glucoside[b,]
60	$C_{27}H_{28}O_{11}$	529	Tremulacin[a]
61	$C_{15}H_{12}O_5$	273	Naringenin (internal standard)
62	$C_{15}H_{10}O_6$	287	Kaempferol[a]
63	$C_{16}H_{12}O_7$	317	Isorhamnetin[a]
64	$C_{18}H_{16}O_8$	361	5.3′.5′-Trihydroxy-6.7.4′-trimethoxyflavone[b], (Kinoshita and Firman 1996)
65	$C_{16}H_{12}O_5$	285	Acacetin[a]
66	$C_{17}H_{14}O_6$	315	Pectolinaringenin[b], (Greenham et al. 2003; Horie et al. 1998; Vieira et al. 2003)
67	$C_{18}H_{16}O_7$	345	Santin[b], (Greenham et al. 2003; Horie et al. 1998)

Compounds were identified using authentic reference substances, by isolation, or by release of the aglycones by enzymatic hydrolysis (see Experimental). Numbering in Table 2 corresponds peak numbering of Fig. 2. [a] compared with reference substances, [b] isolated and compared [1] H and HSQC-NMR and UV, HR-ESI MS spectroscopic data with literature, [c] identification based on the APCI-MS and compared the aglycone with reference substances after hydrolysis, [d] identification based on the APCI-MS and HR-ESI-MS.

larvae were able to sequester salicin from the food plants. Thus, a defect in the transport system does not account for the total lack of salicylaldehyde or other aldehydes (e.g., benzaldehyde from benzylalcohol; see Table 1). Fig. 3 also demonstrates that the thioanalogs of (*Z*)-3-hexenylglucoside, phenylethylglucoside (**42**), and 8-OH-geraniolglucoside (**39**) are easily imported with a similar efficiency. In general, almost identical transport characteristics and capacities were observed for willow- and birch-feeding larvae of *C. lapponica*.

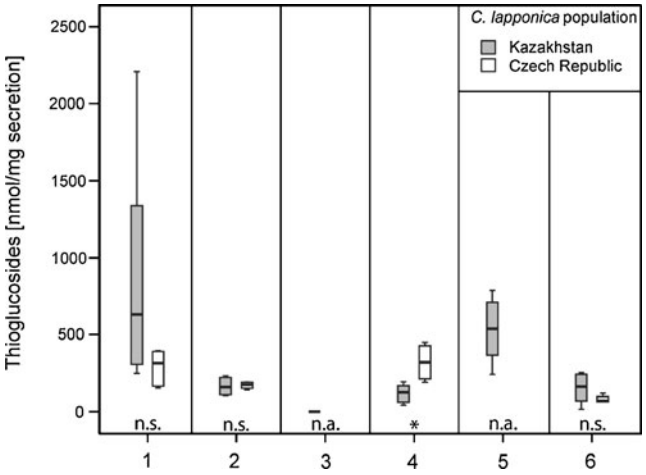


Fig. 3 Accumulation of the thioglucosides **1** to **6** in the defensive secretion of the birch-feeding larvae of *Chrysomela lapponica*. Test compounds: **1**: thiosalicin, **2**: *o*-kresolthioglucoside, **3**: salicylthiogalactoside, **4**: phenylethylthioglucoside, **5**: (*Z*)-3-hexenylthioglucoside, **6**: 8-OH-geraniolthioglucoside. Compounds were determined and quantified by HPLC-MS (see Experimental). Boxplots represent median, 25% and 75% percentile and inner fences show lowest and highest value. Data for larvae collected in the Czech Republic are taken from (Kuhn et al. 2004). According to Mann-Whitney test significant differences were only observed for **4** (P=0.019). No significant differences (n.s.) were observed for **1** (P=0.126), **2** (P=0.914) and **6** (P=0.257). Statistics were not applicable (n.a.) to **3** and **5**

Discussion

Sequestering leaf beetle larvae or those using a mixed-mode biosynthesis (sequestration and *de novo* biosynthesis) have developed an efficient network of transporters in their guts and glandular systems to channel phytogenic precursors into their reservoirs (Kunert et al. 2008; Discher et al. 2009). In general, only the glucoside precursor of the genuine chemical defense (e.g., salicylaldehyde or iridoid) is imported and channeled *via* hemolymph transport through the larval body to the glandular reservoir for the final toxification reactions. For example, larvae of *Chrysomela populi* and *Phratora vitellinae* specifically import salicin (Kuhn et al. 2004) and almost no related compounds. The *de novo* producers *Phratora laticollis*, *Hydrotassa marginella*, *Phaedon cochleariae*, *Gastrophysa viridula*, and *Plagiodera versicolora* also possess the ability to selectively uptake glucosidically bound 8-hydroxygeraniol (Feld et al. 2001; Kuhn et al. 2004) provided that the compound is present in the food plant (Kunert et al. 2008). In all cases, the imported glucosides coincided with the biosynthetic precursors of the final chemical defenses of the defensive cocktail that is based mostly on a single or only a few compounds.

In contrast, *C. lapponica* should possess transport systems of lower selectivity, thus allowing the sequestration of a range of plant-derived glucosides that is reflected in the remarkable pattern of substances within the secretion. Their principal ability to sequester structurally different glucosides was shown previously in *Salix*-feeders from Queyras using hydrolysis-resistant thioglucosides, which remain intact all the way from the leave to the defensive gland (Kuhn et al. 2007). By direct inspection of Tables 1 and 2, which compare the spectrum of glucosides present in the food plant (Fig. 2, Table 2) and the enzymatically released aglycones with the components of the defensive secretion, the selectivity of the sequestration process becomes obvious. For example, neither the dominant flavanoids, nor any of their ester derivatives, accumulate in the defensive secretion. Instead, the aglycones of (*Z*)-3-hexenol, phenylethanol, *p*-OH-phenylethanol, and betuligenol are present as major constituents in the blend of 2-methylbutyryl- and isobutyryl esters. The aglycones are imported into the reservoir as their glucosides, as demonstrated by the successful import of thioglucosides (Fig. 3, similar to the results shown for the Queyras population, (Kuhn et al. 2007). After import into the gland, the sugar moiety is hydrolyzed, followed by acylation of the resulting aglucone with the butyrates. Interestingly, all glucoside precursors of the defensive compounds were present only as minor components in the leaf extract (Fig. 2) suggesting a selective and highly efficient transport process in the larvae.

The complete lack of derivatives of salicortin (**44**) and tremulacin (**60**) in the defensive secretion is striking, although they are present in small amounts in the birch extract. Unspecific esterases, generally occurring in the enzymatic inventory of a gut, could remove the cyclohexenoic acid moiety (Lindroth 1988; Julkunen-Tiitto and Meier 1992), thus generating salicin, which would be readily sequestered into the glandular reservoir (Fig. 3). After hydrolytic cleavage in the reservoir, the released aglycone could be converted into a butyrate or oxidized to salicylaldehyde. Neither reaction was observed. However, the lack of salicylaldehyde or benzaldehyde is readily explained since the birch-feeding population of *C. lapponica* from Kazakhstan has no functional salicylalcohol oxidase (unpublished data).

Additionally, striking is the presence of high amounts of glucose (469 nmol mg^{-1} secretion) and minor amounts of the two glucose-6-*O*-butyrates **27a/b** and **28a/b** in the secretion (44 nmol mg^{-1} secretion). Apparently, glucose not only acts as a carrier for import of plant-derived aglycones into the glandular system, but also may serve as a scaffold for acylation reactions. The same compounds are known from trichomes of tomato (Neal et al. 1990), and an insecticidal activity has been demonstrated (Juvik et al. 1994).

The biological relevance of the high amounts of glucose in the larvae of the Altai populations is not clearly understood, but the sugar apparently does not interfere with the repellent function of the esters, as has been demonstrated (Hilker and Schulz 1994); probably the volatile esters form a repellent plume around the larvae, thus

preventing the necessity of a direct contact, which has been shown previously for *Phratora vitellinae* and *Phaedon cochleariae* (Gross et al. 2008; Gross and Schmidtberg 2009).

On the other hand, since the larvae experience rather low temperatures that go well beyond 0°C at night during the season (July, August), the high amount of glucose may function as a cryoprotectant (Calderon et al. 2009). Moreover, since the defensive secretion is an emulsion of organic esters and water, the actual concentration of free glucose in the aqueous phase, and hence its cryoprotective effect, might be even higher than calculated from the molar concentration (ca. 1.1°C at ca. 470 nmol mg^{-1} secretion). Since in the Altai region predators seem to be rare compared to other habitats of *C. lapponica* (personal observations), the selective pressure on adaptation to climate may be higher than to predation. The risk of infections by microorganism that utilize the "sweet secretion", particularly by entomophagous fungi (e.g., *Beauveria bassiana*) might be reduced by the presence of a few antimicrobially active constituents, such as *p*-OH-phenylethyl-isobutyrate (**19**), *p*-OH-phenylethyl-2-methylbutyrate (**22**), and betuligenoliso-butyrate (**23**) in the secretion (Tolzin-Banasch 2009).

By studying the selective import from the whole spectrum of glucosides that occur in the leaves of the food plant (*B. rotundifolia*), instead of a limited set of model compounds (Discher et al. 2009), we were able to better evaluate the selectivity and efficiency of transport. Although the larvae import several compounds (e.g., **37** or **42**), this corresponds only to a minor fraction of glycosides present in the food plants (Fig. 2). Moreover, the imported compounds do not belong to the major constituents of the plant, thus demonstrating a high enrichment capacity of the transport system. However, *de novo* biosynthesis of certain aglycones, especially phenylethanol and benzylalcohol from phenylalanine (unpublished results) in addition to sequestration cannot be excluded.

Whether or not the transport systems in the two membrane barriers (gut epithelial cells, glandular system) indeed have different selectivities (Discher et al. 2009) remains to be established.

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Task Group Differences in Cuticular Lipids in the Honey Bee *Apis mellifera*

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Abstract Social insects are defined by their ability to divide labor among their numerous nestmates. This is achieved via a complex system of chemical communication that allows colonies to organize task activity so as to maximize the productivity of the colony. However, the mechanism by which social insects distinguish task groups among morphologically identical individuals remains unknown. Using the honey bee, *Apis mellifera*, as our model species, we investigated the presence of task-specific patterns in the cuticular lipids (*n*-alkanes, fatty acids, and alkenes) of bees. Cuticular lipids are known to play an essential role in the recognition processes of insects. We found task-specific features in the *n*-alkane and alkene profiles of bees, but no task-specific patterns in the fatty acid profile. Foragers, in particular, had elevated levels of *n*-alkanes relative to nurse and newly emerged bees, suggesting increased waterproofing. Newly emerged bees had low levels of cuticular lipids, supporting the Blank Slate theory and potentially explaining their acceptance into foreign colonies.

Key Words Cuticular lipids · Recognition · Task · *Apis mellifera*

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Introduction

Social insects owe much of their success to their ability to divide labor, and to their highly sophisticated communication systems. Division of labor requires a high level of colony organization and integration (Hölldobler and Wilson, 1990; Seeley and Seeley, 1995); these processes are underpinned by a multi-layered chemical communication system (Wyatt, 2003).

Cuticular lipids form the waxy layer on an insect's cuticle and are an integral part of an insect's physiology (Lockey, 1988). They typically are composed of esters, alcohols, free fatty acids, and hydrocarbons (*n*-alkanes, alkenes, and branched alkanes), with hydrocarbons often the dominant group. These lipids protect the insect from external influences, such as trans-cuticular water movement and pathogens, but some are also used in communication. As cuticular lipids vary in both quantity and quality (type) among species, age classes, genders, and colonies (Howard and Blomquist, 2005), they have the potential to convey information about an individual's biology.

Even though division of labor is essential to a colony's life, the mechanism by which workers that perform different tasks distinguish each other, especially when they are morphologically similar, as in the honeybee, remains poorly understood. In many social insects, workers performing different tasks have been shown to vary in lipid composition (Howard et al., 1982; Bonavitacougourdan et al., 1993; Wagner et al., 1998; Sevala et al., 2000; Nunes et al., 2009); such studies generally have used multivariate statistics (e.g., Discrimant Analysis and Principal Components Analysis) to show differences. There are two drawbacks to using only multivariate statistics to analyze differences in cuticular lipids among workers performing different tasks. First, such

methods render it difficult to identify task-specific chemical patterns, an important first step for illuminating the precise function of individual compounds. Second, they treat each compound as an independent variable and seek statistical patterns, thus maximizing the variance explained or the among-group component of variation, without reference to chemical relationship or biological function among compounds (Martin and Drijfhout, 2009a). The last point is particularly important, as social insects discriminate and "learn" some lipids more readily than others (Châline et al., 2005; Dani et al., 2005). This implies that lipid profiles revealed by the chemical analysis and those detected by the insects may not be congruent, as not all compounds may be used in communication, but instead serve other functions, such as waterproofing.

Here, we tested the hypothesis that there are differences in cuticular lipids of workers of the European honeybee, *Apis mellifera* L, that perform different tasks. For this, we quantified and compared compounds of the same lipid class, identified task-specific patterns related to these lipid classes, and then tested the potential of these patterns to separate tasks by using multi-variate statistics. We investigated the honeybees' two major cuticular lipid classes, namely *n*-alkanes and alkenes, which consistently occur in the honey bee from emergence onwards and make up the vast majority of the lipid profile (Blomquist et al., 1980). We also included fatty acids, which seem to play a role in the recognition process of the honey bee (Breed, 1998), but have not been quantified on an individual task basis. We compared three task groups: newly emerged bees, foragers, and nurse bees. Although newly emerged bees, strictly speaking, do not form a task group, they allowed us to follow the changes of the lipid profile with age, since a worker's task is age dependent. Choosing three groups had two advantages. First, it allowed us to look at age-dependent changes in cuticular profile from emergence onwards and, second, we were able to quantify changes in cuticular profile when workers experienced environmental conditions different from their hive environment, as in the case of nurse vs. forager bees.

Methods and Materials

Study Organism In October 2007, bees were collected from five queen-right *A. mellifera* colonies. From each colony, 15 newly emerged bees, 15 nurses, and 15 foragers were collected. Since task groups are morphologically indistinguishable, foragers were caught when entering the hive entrance, often with bags of pollen attached to their hind legs, whereas nurses were collected directly from a brood frame containing a high proportion of open brood. Collecting bees at the nest entrance or on the brood frame increased the probability that the majority of bees collected were foragers or nurses, respectively, although a few guard bees waiting at the entrance, or a small number of foragers and young bees sitting on the brood frame, might have been caught as well. Newly emerged bees were collected from a brood frame containing mature sealed brood that was kept in an incubator at 37°C. Newly emerged bees (≤24-h-old) were collected daily. All bees were killed by freezing at −20°C. In total, 196 bees were collected, including 75 foragers, 75 nurses, and 46 newly emerged bees. The lower number of newly emerged bees was because of a lack of sealed brood in two colonies.

Chemical Extraction Cuticular lipids were extracted by immersing each bee in 0.5 ml of HPLC-grade hexane, and then shaking the vial gently at ambient temperature for 2 min. Pollen, if present, was removed from foragers before immersion. After 2 min., the bee was removed from the solvent, and 50 µl of the extract were transferred to a glass insert and left for the solvent to evaporate.

Immediately before analysis, 30 µl BSTFA [N, O-bis (trimethylsilyl) trifluoroacetamide] were added to each sample and left to react at 70°C for 20 min. After silylation, products were extracted in 30 µl hexane and analyzed on an HP 6890GC, equipped with an HP-5MS column (length 30 m, ID: 0.25 mm, film thickness: 0.25 µm; Agilent Technologies Inc., Santa Clara, CA, USA) connected to an HP5973 Mass Selective Detector (electron impact ionization; 70 eV). Samples were injected in the splitless mode. The column oven was programmed from 70°C to 200°C at $40°C.min^{-1}$, and to 320°C at $25°C.min^{-1}$. Helium, at a constant flow rate of 1.0 ml min^{-1}, was the carrier gas.

Compounds were tentatively identified by using standard mass spectral databases, key diagnostic ions, and Kovats indices. Double bond position and geometry was not identified but was assumed to be as in the literature (Blomquist et al., 1980; Breed, 1998). The notation for each compound is based on chain length (e.g., tricosane = C_{23}), and number of double bonds (e.g., pentacosene = $C_{25:1}$).

Statistical Analysis The peak area of each compound was determined by manual integration of each total ion chromatogram (TIC). Compounds that, on average, contributed less than 1% to the overall chemical profile (i.e., *n*-alkanes + alkenes + fatty acids) were excluded from the analysis. Six samples were excluded due to poor chromatography. For each individual, *n*-alkane, fatty acid profile, and alkene profiles were quantified by dividing the peak area of each compound by the total compound abundance of that chemical class per individual; these profiles were compared among the three task groups. A preliminary

analysis indicated that the chemical profile of some individuals in one task group was more similar to that of another task group, and this was consistent across all three chemical classes, presumably because individuals could not be identified as belonging to a particular task group with complete reliability (see above).

All significance tests were performed using the statistical software R (v 2.81). A one-way *ANOVA* was used to test for task group differences in total compound quantity for each compound class, and a two-way *MANOVA*, with *Pillai test* and *post-hoc Tukey test*, was carried out to test for task group differences in chemical profile for each chemical class separately. Data were transformed to log-contrasts and standardized to meet the *MANOVA* assumptions of independence, equal variance, and normal error distribution (Aitchison, 1986). For each chemical class, the least variable compound was used as a reference peak for the log-contrast transformation. C_{27}, $C_{18:1}$, and $C_{29:1}$ were used as reference peaks for *n*-alkanes, fatty acids, and alkenes, respectively. From here on, compounds will be referred to by their names even though the analysis was carried out on each compound relative to the respective reference peak of its chemical class. The newly emerged group was excluded from the *MANOVA* test on the alkenes, as many alkenes were absent or below the detection threshold in this group, rendering the test unstable.

To determine what compound class produced the clearest separation of task groups, a discrimant analysis (DA) was performed for each chemical class using SPSS 13.0. For this, the proportion of each compound (relative to the total compound abundance of that chemical class per individual) was transformed before the multivariate analysis according to the formula (Aitchison, 1986), $Z = ln[A_p/g(A_p)]$ (where A_p is the proportion of the compound and $g(A_p)$ is the geometric mean of all compounds to be included in the multi-variate analysis), so as to avoid complications arising from analyzing compositional data. Each of the 13 task-colony groups (3 newly emerged, 5 nurse, and 5 forager) was treated as a separate group during the DA, so as to investigate the relative separation of task groups and colonies.

Results

Chemical Analysis Five *n*-alkanes, five fatty acids, and six alkenes were identified (Fig. 1). The combination of *n*-alkanes, fatty acids, and alkenes found in this study agreed with previous studies (Blomquist et al., 1980; Carlson, 1988; Dani et al., 2004). All *n*-alkanes and alkenes had an odd number of carbons in the chain, with lengths ranging from 23 to 33. In contrast, fatty acids had an even number of carbons in the chain, from 16 to 28.

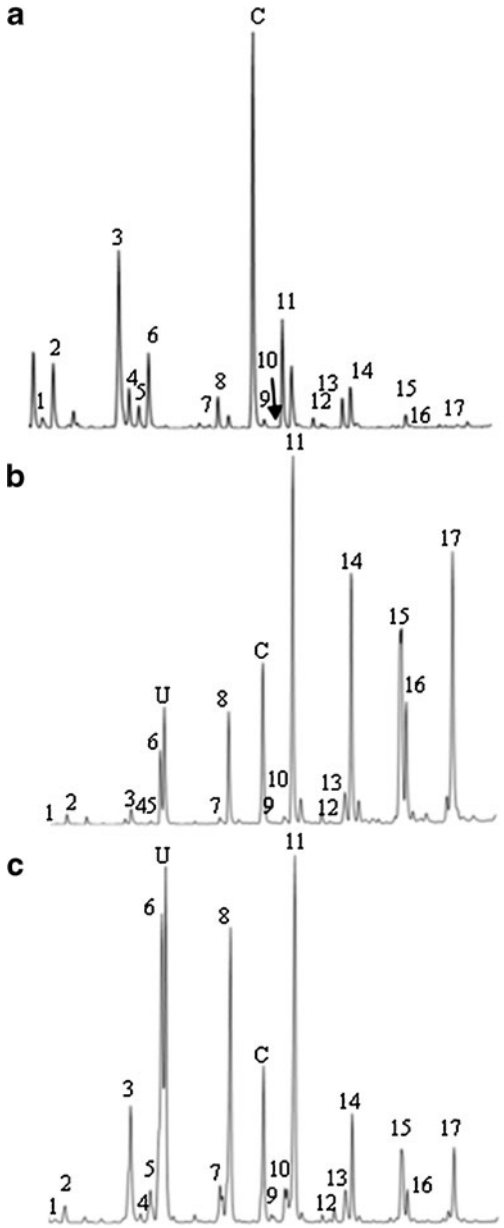


Fig. 1 Gas chromatogram of **a**) newly emerged, **b**) nurse, and **c**) forager honey bee. 1 = palmitoleic acid ($C_{16:1}$); 2 = palmitic acid ($C_{16:0}$); 3 = oleic acid ($C_{18:1}$); 4 = stearic acid ($C_{18:0}$); 5 = tricosene ($C_{23:1}$); 6 = tricosane (C_{23}); U = eicosenol, 7 = pentacosene ($C_{25:1}$); 8 = pentacosane (C_{25}); C = contaminating phalate; 9 = docosanoic acid ($C_{22:0}$); 10 = heptacosene ($C_{27:1}$); 11 = heptacosane ($C_{27:0}$); 12 = lignoceric acid ($C_{24:0}$); 13 = nonacosene ($C_{29:1}$); 14 = nonacosane ($C_{29:0}$), 15 = hentriacosene ($C_{31:1}$); 16 = hentriacosane (C_{31}); 17 = tritriacosene ($C_{33:1}$)

Although there was a significant overall colony effect of the *n*-alkanes (*MANOVA*, F=9.92, *d.f.*=16, 147, P< 0.001, *Pillai*), fatty acids (*MANOVA*: F=6.05, *d.f.*=16, 147, P<0.001), and alkenes (*MANOVA*: F=10.11, *d.f.*= 16, 147, P<0.001, *Pillai*), further analysis using Tukey multiple comparison tests indicated that this arose due to one or two colonies having a chemically distinct group of

newly emerged forager, or nurse bees, e.g., when analyzing $C_{25:1}$, foragers of colony 1 differed from foragers of colony 2 ($P<0.001$) and colony 3 ($P<0.001$), although there was no significant difference ($P>0.08$ in all cases) among foragers of colonies 2, 3, 4, and 5. However, the nurses of colony 1 were not different ($P>0.8$) from nurses of any other colony, while nurses of colony 4 were different from nurses of colonies 2, 3, and 5 ($P<0.01$). This inconsistent pattern was found across almost all of the 16 compounds analyzed (Fig. 1), and is not indicative of a colony signal that one would expect to be consistent across all nurses and foragers from the same hive. We found that in the majority of cases, different age groups (task groups) of the same colony were different (*Tukey test*: $P<0.01$ in all cases), whereas groups of the same age, but from different colonies, were not (*Tukey test*: $P>0.05$). Therefore, we concluded that there was no consistent colony effect for any of the three chemical classes.

Differences in Quantity The three task groups differed considerably (*ANOVA*: *d.f.*=2, $P<0.001$) in amount of *n*-alkane produced, with the amount increasing with age (Fig. 2a). Foragers produced around 70% more *n*-alkane compared to nurses, and 1,000% more than newly emerged bees. Fatty acid quantity also increased (*ANOVA*: *d.f.*=2, $P<0.01$) from newly emerged to nurse bees (Fig. 2b), but there was no difference (*ANOVA*: *d.f.*=2, $P=0.90$) between nurse bees and foragers. Task groups also differed (*ANOVA*: *d.f.*=2, $P<0.001$) in the amount of alkene produced. Alkene quantity was extremely low in newly emerged bees, increased by 1,300% at the nurse stage, and then dropped by 40% for foragers (Fig. 2c).

Differences in Chemical Profile The three task groups varied in overall chemical profile (Fig. 1). By considering the three chemical classes separately, it can be seen that *n*-alkanes effected the clearest separation of task groups (Fig. 3a). Newly emerged bees, nurses, and foragers clustered into distinct groups, independent of colony origin, with little overlap among them (Fig. 3a). In contrast, according to fatty acid profiles, bees formed a single cluster with no task group separation (Fig. 3b). Nurses and foragers also could be separated on alkene profiles (Fig. 3c). The newly emerged group was removed from this analysis because many alkenes were not detected in members of this group (Fig. 3c).

A clear task group separation also was found in the actual chemical profiles of bees when based on *n*-alkanes and alkenes, as opposed to fatty acids (Fig. 4). The three age groups varied little in fatty acid profile (Fig. 4a–c), leading to the poor group separation of Fig. 3b. All groups were dominated by palmitic ($C_{16:0}$) and oleic ($C_{18:1}$) acids. The increased amount of lignoceric acid ($C_{24:0}$) observed in the nurse profile was evident only in two of five colonies and, thus, the overall task group effect was not significant (*MANOVA*: $F=14.06$, *d.f.*=12, 147, $P=0.08$). Groups differed, however, in their *n*-alkane and alkene profiles. The clearest difference among groups was in the profiles of compounds within a chemical class. Newly emerged bees and nurses (Fig. 4a, b) showed similar *n*-alkane and alkene profiles, with the main difference being that newly emerged bees had relatively higher levels of $C_{23}/C_{23:1}$, whereas nurses produced relatively more C_{31}/C_{29} and $C_{30:1}$ and $C_{33:1}$ (Tukey multiple comparison test, $P<0.001$). The high $C_{23:1}$ peak observed in the newly emerged bee profile is an artifact of the absence of other alkenes in many of the young bees, and this was only observed at this extreme in one colony. The *n*-alkane and alkene profiles of foragers did not show similar (to the other groups) patterns (Fig. 4c). The relative increase of C_{23}/C_{25} and decrease in C_{29}/C_{31} rendered foragers chemically distinct (*Tukey test*, $P<0.001$) from nurses and newly emerged bees. At the same time, the increase (*Tukey test*, $P<0.001$) of $C_{23:1}/C_{25:1}$ and decrease of $C_{30:1}$ and $C_{33:1}$ lead to a relatively even abundance profile of alkenes in foragers.

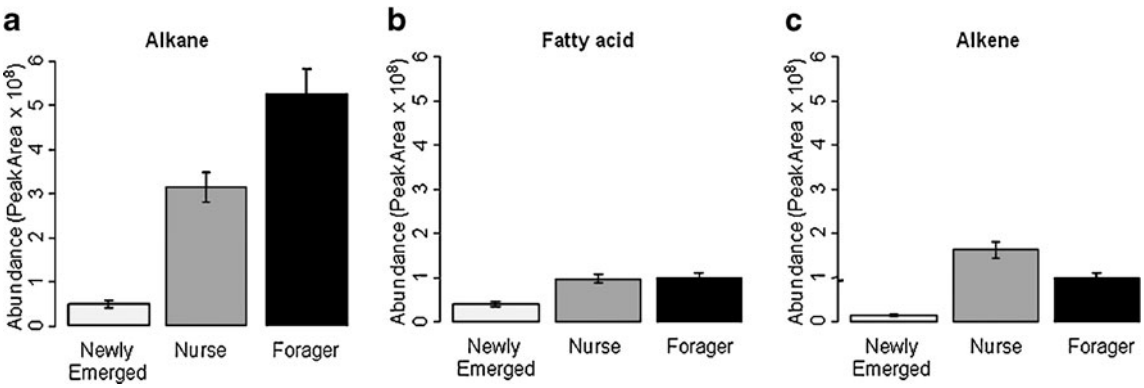


Fig. 2 Total abundance of: **a**) *n*-alkane, **b**) fatty acid and **c**) alkene produced by newly emerged honey bees (light grey bars), nurses (dark grey bars), and foragers (black bars). Error bars are standard errors

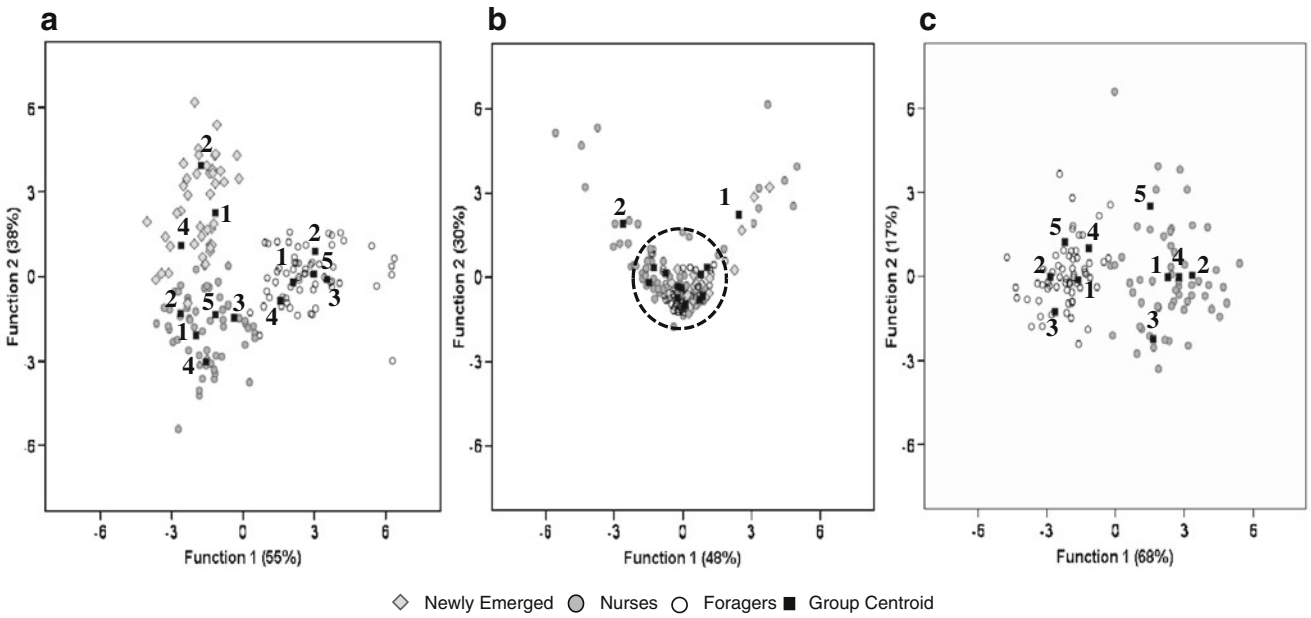


Fig. 3 Caste separation based on discriminant analysis (DA) of a honey bee's **a**) *n*-alkane, **b**) fatty acid, and **c**) alkene profiles. For clarity, the centroid of each group was given its colony number (1 to 5) to show colony separation as well as caste separation. In the fatty acid DA (3b), all groups except the nurses of colony 2 and 1 are contained in the dashed circle. The proportion of variance explained by each function is given in brackets

Discussion

We demonstrated that *A. mellifera* task groups differed in their chemical profiles in at least two of the three compound classes investigated, namely *n*-alkanes and alkenes. Changes in cuticular chemical profiles of insects can occur during development, for example, such as reaching sexual maturity (Nelson et al., 1981), or can be affected by changes in temperature and humidity (Gibbs et al., 1991; Wagner et al., 2001). The most dramatic change we observed in the *n*-alkane profile of *A. mellifera* occurred during the transition from nurse to forager, as has been previously reported (Dani et al., 2004; Del Piccolo et al., 2010). During this transition, a honey bee's duty changes from working within, to foraging outside, of the hive. We found that this change was accompanied by a distinct increase in *n*-alkane quantity and a change in the *n*-alkane profile. This change in hydrocarbon profile could be a response to the change in temperature and humidity levels that the bee is exposed to when leaving the hive and may provide better waterproofing under more variable conditions.

In general, high levels of cuticular *n*-alkanes typically are associated with species living in hot, arid habitats (e.g., Hadley and Schultz, 1987). *n*-Alkanes are thought to be the best class of hydrocarbon for providing cuticle waterproofing, because of their high melting point and shape (Gibbs and Pomonis, 1995; Gibbs, 1998). In several species of ants, foragers produce significantly higher amounts of the *n*-alkanes C_{25} and C_{27} than do nest-maintenance workers (Wagner et al., 1998; Martin and Drijfhout, 2009b). The red harvester ant (*Pogonomyrmex barbatus*) uses this difference to distinguish workers performing different tasks (Greene and Gordon, 2003). Wagner et al. (2001) showed that the increase in *n*-alkane quantity is caused by exposing a nest-maintenance worker for just 50 min to a higher temperature (>27°C) and lower humidity (<43%) than conditions found in the nest. Furthermore, several species of flies, including *Sargophala bullata*, *Musca domestica*, and *Pteronarcys californica,* increase levels of alkanes in response to dry and warm conditions (Armold et al., 1969; Noorman and Den Otter, 2002).

To what extent, if at all, honeybees use *n*-alkane dimorphism to distinguish between nurses and foragers remains to be tested. Châline et al. (2005) found that honey bees detect and remember alkenes more readily than they do *n*-alkanes. Dani et al. (2005) and Breed (1998) showed that changes in a bee's *n*-alkane profile did not change how the bee was treated by nest mates, supporting the theory that *n*-alkanes are probably used more for waterproofing than communication. In contrast to this, changes in a bee's alkene (Breed et al., 1998; Dani et al., 2005) and fatty acid profiles (Breed et al., 1998) resulted in a bee being attacked by its own nestmates, implying that these compounds may serve in communication, especially as alkenes are believed to be poor waterproofing agents (Gibbs, 1995). Dani et al. (2005) and Breed and Stiller (1992) suggested that alkenes and fatty acids may be used by bees to recognize nestmates. In our study, task groups varied in alkene profile, suggesting

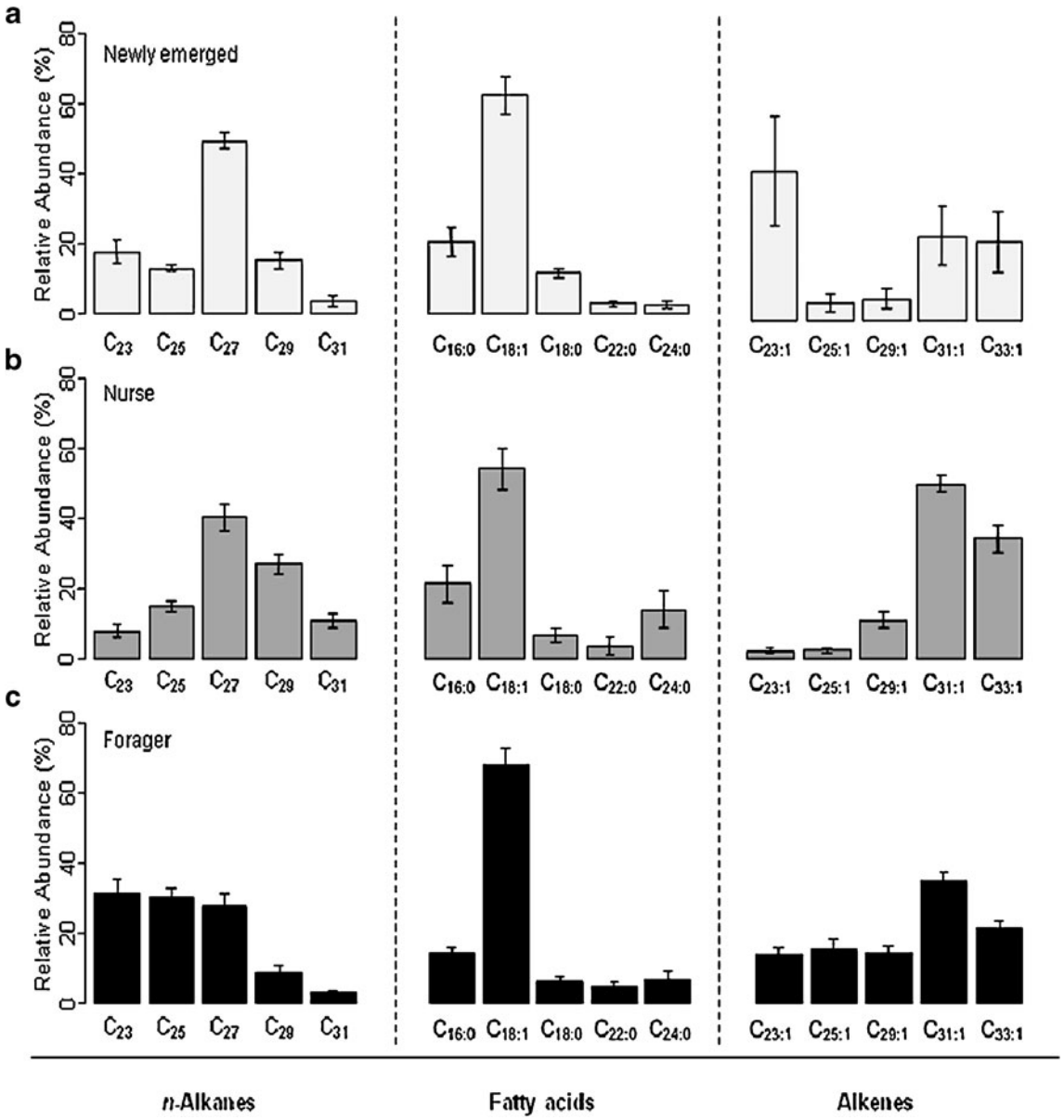


Fig. 4 Differences in chemical profiles of different task groups [newly emerged = light grey bars; nurses = dark grey bars; foragers = black bars] of honey bees. Relative abundance of **a**) *n*-alkanes (C_{23} = tricosane; C_{25} = pentacosane; C_{27} = heptacosane; C_{29} = noncacosane; C_{31} = heintriacontane), **b**) fatty acids ($C_{16:0}$ = palmitic acid; $C_{18:1}$ = oleic acid; $C_{18:0}$ = stearic acid; $C_{22:0}$ = docosanoic acid; $C_{24:0}$ = lignoceric acid), and **c**) alkenes ($C_{23:1}$ = tricosene; $C_{25:1}$ = pentacosene; $C_{29:1}$ = nonacosene; $C_{31:1}$ = hentriacosene; $C_{33:1}$ = tritriacosene). Error bars are standard errors

that bees could use these alkenes to distinguish task groups; this will need to be investigated. However, we did not find a difference in alkene profile among colonies, contradicting the hypothesis of Dani et al. (2005). Fatty acids, also, did not contain sufficient variation to separate task groups or colonies, suggesting that these acids do not function in task group or colony recognition; the latter contradicts the findings of Breed and Stiller (1992).

Nevertheless, there are two explanations for a lack of colony effect of alkenes and fatty acids. First, queens of the colonies used in this study may have been related, which could result in reduced chemical variation among colonies. Second, alkenes and fatty acids contain another level of information in the form of isomers. In *A. mellifera*, alkenes of a similar chain-length contain several isomers with double bonds at carbons 7, 8, 9, 10, or 12 (Dani et al., 2004). Breed et al. (2004) reported only one isomer of octadecenoic (with a double-bond at carbon 9; i.e., oleic) acid, but reported two polyunsaturated fatty acids, (*Z*, *Z*)-9,12-octadecenoic acid and (*Z*,*Z*,*Z*)-9,12,15-octadecatrienoic acid; these polyunsaturated acids were present in quantities below the detection limit of our method. These

alkene and fatty acid isomers may contain sufficient variation to separate colonies. A recent study (Martin et al., 2010) found that bumblebees have a high diversity of alkene isomers, of which some are mimicked by bumblebee parasites, thereby enabling them to integrate into the host colony. Furthermore, newly emerged bees are readily accepted into colonies if they lack essential nestmate recognition compounds; this is known as the Blank Slate Theory proposed by Breed et al. (2004). A lower amount of cuticular lipids at this early developmental stage appears to be common in bees (Breed et al., 1988, 2004), wasps (Panek et al., 2001), and ants (Lenoir et al., 2001). In our study, the class of compounds found in the lowest quantities was alkenes, followed by fatty acids, and then *n*-alkanes. This suggests that alkenes may be the most likely compound group to function as colony recognition compounds. However, this needs investigation.

In light of our results, it remains to be tested whether the task-group differences in *n*-alkane profiles are indeed adaptations to outside conditions and whether or not honey bees use these compounds to distinguish task groups. Further investigations are also needed into the role of alkenes as task- and colony-recognition compounds, and into any role fatty acids may play in these processes.

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Alkaloids in the Mite *Scheloribates laevigatus*: Further Alkaloids Common to Oribatid Mites and Poison Frogs

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Abstract Poison frogs are chemically defended from predators by diverse alkaloids, almost all of which are sequestered unchanged from alkaloid-containing arthropods in the frog diet. Oribatid mites recently have been proposed as a major dietary source of poison frog alkaloids. Here, we report on alkaloids common to an oribatid mite and poison frogs. Gas chromatographic-mass spectrometric analysis of methanol extracts of adult *Scheloribates laevigatus* (Oribatida: Scheloribatidae) revealed nine alkaloids. Five of these have been detected previously in the skin glands of poison frogs: two isomers of the pumiliotoxin **291G**, two isomers of the 5,6,8-trisubstituted indolizidine **209C**, and the 5,6,8-trisubstituted indolizidine **195G**. The other four alkaloids, a pumiliotoxin, a tricyclic (coccinelline-like), and two isomers of an izidine, were not previously known, but are similar in structure to alkaloids found in poison frogs. Alkaloids were not detected in immature *S. laevigatus*, suggesting that they are adult-specific and possibly the result of mite biosynthesis. Although most of the alkaloids detected in *S. laevigatus* are common to poison frogs, the geographic distributions of these organisms are not sympatric. The findings of this study indicate that oribatid mites, and in particular, members of the genus *Scheloribates*, represent a relatively unexplored arthropod repository for alkaloids and a significant dietary source of alkaloids in poison frogs.

Key Words Chemical defense · Dendrobatids · Indolizidines · Oil glands · Opisthonotal glands · Pumiliotoxins · Scheloribatidae · Tricyclic alkaloids · Poison frog

Introduction

The term ‘poison frog’ refers to the four known evolutionary lineages of anurans characterized by the ability to sequester alkaloid-based chemical defenses from dietary arthropods. They include certain dendrobatids from Central and South America, mantellids from Madagascar (*Mantella*), bufonids from South America (*Melanophryniscus*), and myobatrachids from Australia (*Pseudophryne*). Over the past 40 years, more than 850 lipophilic alkaloids, organized into over 20 structural classes, have been detected in the skin glands of poison frogs, apparently reflecting the diversity of alkaloids present in arthropod prey (Daly et al., 2005; Saporito et al., 2009). Most of these alkaloids are pumiliotoxins, indolizidines, and coccinelline-like tricyclics (Saporito et al., 2009), and have molecular weights of less than 400 atomic mass units (Daly et al., 2005). A large number of alkaloids have been identified in arthropods (Jones and Blum, 1983; Braekman et al., 1998), and insofar as is currently known, most poison frog alkaloids are sequestered from alkaloid-containing mites, ants, beetles, and millipedes in the diet (Saporito et al., 2009).

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Oribatid mites are among the most abundant and diverse arthropods in soil and leaf-litter, both in temperate and tropical regions (Maraun and Scheu, 2000; Franklin et al., 2004), but they also are found frequently in arboreal microhabitats, both on exposed surfaces and in "suspended soil" (Lindo and Winchester, 2006). Their food generally consists of decaying higher plant material and saprophytic fungi (Schneider and Maraun, 2005), although stable isotope studies indicate that necrophagy or predation on small invertebrates also is common, especially in tropical soils (Illig et al., 2005). Consistent with low rates of secondary production and relatively long lives (1–5 years or more), these mites have multiple defenses, especially as adults (Norton, 1994). These defenses include cuticular sclerotization or mineralization, specialized cuticular outgrowths, special defensive body forms and behaviors, and defensive chemicals (Sanders and Norton, 2004; Schneider and Maraun, 2009). Chemical defenses probably are derived from paired exocrine glands (Raspotnig et al., 2003) called opisthonotal or oil glands. These glands secrete a wide range of organic compounds including monoterpenes, sesquiterpenes, aromatics, aliphatic aldehydes, a ketone, fatty acids, fatty acid esters, an alkyl formate, and hydrocarbons (Kuwuhara, 2004; Raspotnig, 2009).

Recently, alkaloids have been identified in oribatid mites, representing a repository of these natural products in animals as well as a major dietary source for these compounds in poison frogs (Takada et al., 2005; Saporito et al., 2007a, 2009). More than 80 alkaloids, representing 11 structural classes, have been identified in oribatids, including pumiliotoxins, a homopumiliotoxin, 5,8-disubstituted- and 5,6,8-trisubstituted indolizidines, dehydro-5,8-disubstituted indolizidines, coccinelline-like tricyclics, a 1,4-disubstituted quinolizidine, a 4,6-disubstituted quinolizidine, a 3,5-disubstituted indolizidine, two pyrrolidines, a spiropyrrolizidine, and several that could not be assigned to a specific structural class (Saporito et al., 2009). These alkaloids have been reported in the scheloribatid mites *Scheloribates azumaensis* and *Scheloribates* sp. from Japan (Takada et al., 2005), and in oribatid mites from Costa Rica and Panama, including numerous scheloribatid mites (Saporito et al., 2007a). The full extent of the diversity and taxonomic distribution of alkaloids in oribatid mites is unknown, but initial studies indicate that they are common in the widespread family Scheloribatidae.

As part of a large, ongoing study on the taxonomic distribution of alkaloids in oribatid mites, we analyzed extracts from adult and immature specimens of the soil mite *Scheloribates laevigatus* (C. Koch) (Acari: Oribatida: Scheloribatidae). This mite is a generalist feeder on fungi, algae, and litter (Woodring and Cook, 1962; Hubert et al., 2001) and is common and abundant in Europe and in anthropogenic habitats in North America, where it appears to be an introduced species (Marshall et al., 1987; Weigmann, 2006; R. Norton, unpublished). Here, we report the presence of pumiliotoxins, indolizidines, and a coccinelline-like tricyclic alkaloid in adults of this species. Most of these alkaloids have been detected previously in the skins of poison frogs, lending support to suggestions that scheloribatid mites are an important dietary source of alkaloids in poison frogs.

Methods and Materials

Mite Collection and Sample Preparation Adult mites were extracted with Berlese-funnels from soil and leaf-litter samples collected near Syracuse, NY, USA. Individuals were maintained at room temperature in small glass jars containing a plaster-charcoal substrate, and were provided with alga-covered tree bark and small amounts of leaf-litter and woody debris. Reproduction in the cultures provided a source of immature mites. For chemical analysis, adult or immature mites (10–25 individuals) were extracted for 24 hr in glass vials with 20 µl of methanol.

Alkaloid Identification When possible, alkaloids were identified by comparison of mass spectra, vapor-phase FTIR spectra, and GC retention times to those of previously reported poison frog alkaloids (Daly et al., 2005). Poison frog alkaloids are assigned code names consisting of a bold-faced number corresponding to the nominal mass (molecular weight) and a bold-faced letter to distinguish alkaloids with the same nominal mass (Daly et al., 2005).

Alkaloids in *S. laevigatus* were quantitated by extracting 25 adult mites with 20 µl of methanol containing 100 ng of nicotine [(-)-nicotine ≥99%, Sigma-Aldrich, Milwaukee, WI, USA] as an internal standard. Nicotine was used because its retention time is outside of the typical values for most mite alkaloids. All alkaloids were quantitated by comparing peak areas to that of the nicotine internal standard in the total ion current (TIC) gas-chromatogram (see *Chemical Analysis*) with the ICIS peak detection function in Xcalibur © version 1.4 SR1 (Thermo Electron Corporation, 1998–2003). Although a flame ionization detector (FID) may provide better dynamic range linearity, the moderate precision reported in this paper can be achieved with either TIC or FID if an internal standard is used to account for losses due to instrument behavior and sample manipulation. A similar analysis was done for immature mites, but without the internal standard, which could interfere with the determination of low levels of alkaloids. The analyses were repeated on three independent extracts.

Chemical Analysis GC-MS data were obtained on a Thermo-Electron Polaris-Q instrument coupled to a Focus GC with a 30 m×0.25 mm i.d. Restek-5MS fused silica column. GC separation of alkaloids was achieved using a temperature program from 100 to 280°C at a rate of 10°C per min with He as carrier gas (flow rate: 1 ml/min.). Each extract was analyzed with both electron impact-mass spectrometry (EI-MS) and chemical ionization-mass spectrometry (CI-MS) with NH_3 as the reagent gas. High resolution GC-MS analysis was obtained using a Waters GCT instrument coupled to a Hewlett-Packard model 6890 GC, with a column, temperature program, and carrier gas conditions identical to those listed above. GC-FTIR data were obtained with a Hewlett-Packard model 5890 gas chromatograph fitted with a 30 m×0.32 mm i.d. Phenomenex Zebron ZB-5 capillary column (same temperature program as above), interfaced with a Hewlett-Packard model 5971 Mass Selective Detector and a Model 5965B IRD with a narrow range (4000–750 cm^{-1}) infrared detector.

Results and Discussion

GC-MS analysis of adult *Scheloribates laevigatus* extracts detected nine alkaloids (including isomers), representing five structural classes (Figs. 1 and 2a; Table 1). Five of these have been identified previously in the skin of poison frogs: the 5,6,8-trisubstituted indolizidines (5,6,8-I) **195G** and **209C** (two isomers) and pumiliotoxin (PTX) **291G** (two isomers). The 5,6,8-I **195G** also has been previously detected in an unidentified oribatid mite species in the family Scheloribatidae from Bocas del Toro, Panama

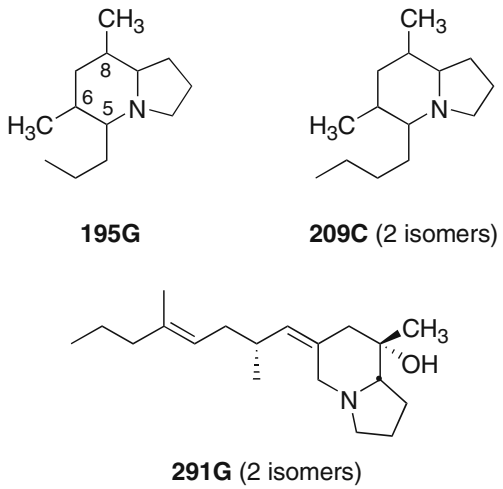


Fig. 1 Structures of alkaloids identified in *Scheloribates laevigatus* that have been previously identified from the skins of certain poison frogs

(Saporito et al., 2007a). The remaining four alkaloids have not been observed previously, but are similar in structure to other alkaloids identified in poison frogs: a pumiliotoxin of molecular weight (MW) 307, a tricyclic (Tri) of MW 247, and an izidine (two isomers) of MW 275 (Table 1; see Supplementary Material for vapor-phase FTIR). Tricyclics represent a large alkaloid group of varied structural types, characterized by mass spectra with many intense peaks, often homologous, but without any dominant alpha-substituent cleavages, probably having three aliphatic rings, and are typified by the coccinelline alkaloids (Daly et al., 2005). Izidines also are a large alkaloid group of varied structural types that are generally characterized as bicyclic izidines with either one or two substituents readily lost by alpha-cleavage during mass spectrometric analysis, but without fragmentation patterns corresponding to any of the currently known pyrrolizidine, indolizidine, or quinolizidine classes (Daly et al., 2005). The chemical properties (MW, empirical formulae, GC retention times, mass spectral data, vapor-phase FTIR spectral data, and other data) of these previously unreported alkaloids are found in Table 1. Additional physicochemical characterizations (e.g., ^{1}H and ^{13}C NMR) will be necessary in order to assign definitive structures to these alkaloids.

Nine frog-skin alkaloids of MW 275 are currently known with the empirical formula assigned to the two new MW 275 mite alkaloids. They are assigned codes of **275A-G**, **275I**, and **275K** and are found in dendrobatid, mantellid, or bufonid species. None has a mass spectrum similar to the ones from the two mite MW 275 isomers. Nine dendrobatid or mantellid frog alkaloids, **247B-247J,** have MW 247 and the same empirical formula calculated for the mite MW 247 alkaloid. Only one of the frog compounds is a tricyclic, and it has a significantly different mass spectrum from that of the mite alkaloid.

Individual *S. laevigatus* adults (average wet mass 29 μg) contained approximately 17±2 ng (mean ± SD) of alkaloids, about 0.06% of the mite's body weight. The most abundant alkaloid was PTX **291G** (two isomers), accounting for approximately 9 ng (50%) of the total alkaloid quantity per mite. Takada et al. (2005) reported that individual *S. azumaensis* contained about 10 ng of PTX **251D**. The next most abundant alkaloids in *S. laevigatus* were the novel alkaloids PTX of MW 307, Tri of MW 247, and the two izidines of MW 275 (all of them present in approximately equal amounts), followed by the 5,6,8-Is **195G** and **209C** (two isomers). Figure 2a is a representative chromatogram illustrating the relative alkaloid quantities for adult *S. laevigatus*.

All of the alkaloids identified in *S. laevigatus* contain branch points in their carbon skeleton (Fig. 1), which appears to be the most common alkaloid structural characteristic seen in oribatid mites (Saporito et al., 2009).

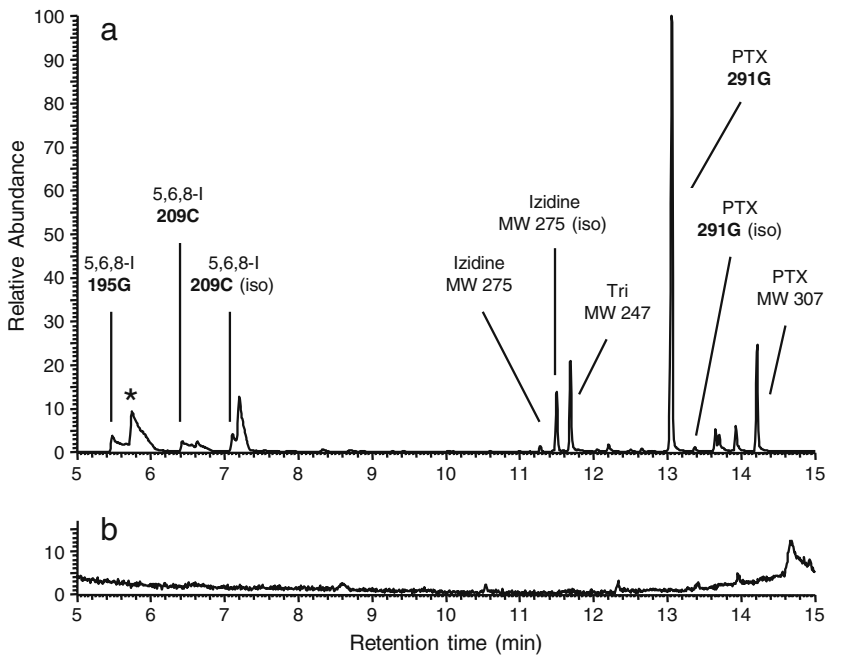


Fig. 2 Gas chromatogram of *Scheloribates laevigatus* extracts in adults (**a**) and immature mites (**b**). The unlabeled peaks in chromatogram a and b are not alkaloids. 5,6,8-I=5,6,8-trisubstituted indolizidine; PTX = pumiliotoxin; Tri = coccinelline-like tricyclic. An *asterisk* (*) indicates the nicotine internal standard in the adult extracts. The term (*iso*) refers to an alkaloid isomer

Numerous branched-chain alkaloids (including pumiliotoxins, 5,6,8-trisubstituted indolizidines, and tricyclics) have been identified previously in oribatid mites, including other members of the genus *Scheloribates* (Saporito et al., 2009). Takada et al. (2005) identified the branched-chain alkaloids PTX **251D**, Tri **193C** (precoccinelline), and an unidentified Tri in *Scheloribates azumaensis*, and the branched-chain PTX **237A**, deoxy-pumiliotoxin **193H**, 5,6,8-I **223A**, and the 1,4-disubstituted quinolizidine (1,4-Q) **231A** in an unidentified *Scheloribates* sp. from Japan. Saporito et al. (2007a) identified mainly branched-chain alkaloids in several different species of oribatid mites (including *Scheloribates*) from Costa Rica and Panama, which included pumiliotoxins, a homopumiliotoxin, 5,6,8-trisubstituted indolizidines, 5,8-disubstituted indolizidines (5,8-Is), dehydro-5,8-disubstituted indolizidines, a 1,4-disubstituted quinolizidine, tricyclics (including precoccinelline), and a spiropyrrolizidine (see Tables 1 and 2 in Saporito et al. 2007a, 2008). A smaller number of unbranched alkaloids also have been identified in oribatid mites (Saporito et al., 2007a). Branched-chain alkaloids also represent the most common alkaloid structural type found in poison frogs worldwide, accounting for approximately 70% of all classified alkaloids, the majority of which are pumiliotoxins, 5,6,8-Is, 5,8-Is, and tricyclics (Saporito et al., 2009).

GC-MS analysis indicated that immature *S. laevigatus* contained no detectable alkaloids (Fig. 2b), which suggests that alkaloids are adult-specific and possibly biosynthesized by the mites. Under culture conditions, both adults and immature mites had the same food choices, and therefore it is unlikely that adult *S. laevigatus* acquire alkaloids or precursor(s) from a dietary source. The culture substrates provided to the mites—tree bark with *Protococcus* alga and secondary fungal growth—are not known to contain the alkaloids in question. However, it remains possible that: (1) adults contained alkaloids that were sequestered from a natural diet, whereas immature mites, which bred in culture, did not have an opportunity to sequester such alkaloids from a natural diet; (2) adults and immature mites had unobserved dietary differences within the cultures; and/or (3) immature mites lack an ability to acquire alkaloids or precursor(s) from their diet. Takada et al. (2005) similarly

Table 1 Chemical properties of previously unreported alkaloids from oribatid mites. These alkaloids have not been found in poison frogs

247. Tri. $C_{17}H_{29}N$. Rt 12.24. MS: *m/z* 247 (18), 232 (5), 218 (12), 204 (22), 190 (100), 176 (78), 162 (52), 148 (36), 137 (50), 136 (35), 124 (16), 96 (22), 70 (20). Vapor phase FTIR (cm^{-1}): 3015 (cis double bond), 2968, 2938 (s), 2866, 2791 (strong Bohlmann band), 984. DBE = 4.

275. Izidine. $C_{19}H_{33}N$. Rt 11.84. MS: *m/z* 275 (22), 246 (5), 190 (48), 178 (22), 150 (100), 136 (20), 70 (8). DBE = 4.

275. Izidine (Isomer). $C_{19}H_{33}N$. Rt 12.06. MS: *m/z* 275 (20), 246 (5), 190 (55), 178 (24), 150 (100), 136 (18), 70 (10). Vapor phase FTIR (cm^{-1}): 2967(s), 2901, 2789. DBE = 4.

307. PTX. $C_{19}H_{33}NO_2$. Rt 14.78. MS: *m/z* 307 (<1), 291 (15), 206 (38), 194 (12), 193 (14), 176 (16), 166 (100), 70 (35). Vapor phase FTIR (cm^{-1}): 3543 (H-bonded OH), 2969 (s), 2888, 2799 (strong Bohlmann band), complex fingerprint region typical of PTXs. DBE = 4.

The tabulation of alkaloids and format of properties, including the Rt values which were proportionally corrected to the known value, follow those of the Appendix in Supporting Information of Daly et al. (2005)

reported that alkaloids occur only in adults of *S. azumaensis* and *Scheloribates* sp., and suggested that alkaloids may be biosynthesized by adult oribatid mites. Our study supports the hypothesis that adult oribatids synthesize alkaloids, but the possibility of a microsymbiont cannot be excluded. Microsymbionts are the source of chemical defenses and/or natural products in a variety of organisms, including vertebrates and invertebrates (Piel, 2002; Daly, 2004).

Some of the alkaloids (and alkaloid classes) found in oribatid mites have been identified in other arthropods. These include some pumiliotoxins, which have been detected in formicine ants of the genera *Brachymyrmex* and *Paratrechina*; some tricyclics (including precoccinelline), detected in coccinellid beetles; a spiropyrrolizidine, detected in polyzoniid millipedes in the genera *Rhinotus* and *Kiusiozonium*; and a number of unbranched-chain alkaloids that were originally considered to be exclusively of myrmicine ant origin: a 3,5-disubstituted indolizidine, two 4,6-disubsituted quinolizidines, and two pyrrolidines (Saporito et al., 2009).

The occurrence of identical alkaloids in both oribatids and other arthropods raises the possibility that some may be transferred among different arthropod groups, possibly through trophic interactions (Takada et al., 2005; Saporito et al., 2009). Certain myrmicine ants in the genera *Myrmecina*, *Pheidole*, and *Oligomyrmex*, and several families of small beetles (Ptiliidae, Staphylinidae, and especially Scydmaenidae) are predators of oribatid mites (Ito and Takaku, 1994; Sanders and Norton, 2004; Wilson, 2005). Furthermore, oribatid mites can be found in ant nests as specialized or unspecialized myrmecophiles (Ito and Takaku, 1994). Although it is not known if alkaloids are present in arthropod predators of oribatid mites, it is conceivable that predation and sequestration could explain the presence of identical alkaloids in at least some of these evolutionarily distinct arthropod lineages. However, it is also possible that different lineages of arthropods synthesize identical alkaloids, and/or that there is a yet unknown, widespread alkaloid-containing microsymbiont present among different arthropod groups.

The biological function of alkaloids in *S. laevigatus* and other oribatid mites is unknown. However, the pumiliotoxins, which comprise the most abundant alkaloids in *S. laevigatus*, are highly toxic (Weldon et al., 2006) and may defend oribatid mites against predation. In poison frogs, alkaloids serve as a chemical defense against potential invertebrate and vertebrate predators (Saporito et al., 2007b), microorganisms (Macfoy et al., 2005), and possibly ectoparasites (Weldon et al., 2006). The functions of other compounds identified in oribatid mites have not been fully studied, but include alarm signals and chemical defenses (Shimano et al., 2002; Raspotnig et al., 2003). In the better-studied and closely related mite group Astigmata, organic compounds also function as aggregation signals and sex pheromones (Kuwuhara, 2004). Alkaloids most likely comprise part of the defensive "tool box" of adult *S. laevigatus*, similar to the non-alkaloid secretions present in immature mites of this species and in many other oribatid mites (Raspotnig, 2009). Research is needed to identify the specific role of alkaloids in oribatids, and to determine why there is such a marked ontogenetic shift in scheloribatid mite gland chemistry, if indeed the alkaloids are housed there.

It has been established from analysis of stomach contents that mites constitute a major prey item for many poison frogs (Simon and Toft, 1991; Valderrama-Vernaza et al., 2009). It is evident that some oribatid mites contain a relatively high diversity and quantity of alkaloids, and that many of these are in the skin of poison frogs. We think oribatid mites may constitute the most important dietary source of alkaloids in poison frogs, more so than ants, which have long been considered to be the principal source (Saporito et al., 2004). Furthermore, since members of the widespread genus *Scheloribates* contain the greatest diversity of alkaloids known from oribatids to date, scheloribatids may be an especially important dietary item for poison frogs worldwide. Additional studies of poison frog diet through stomach content analysis, in which mites are taxonomically identified, coupled with information on the distribution and availability of scheloribatids, will be necessary to understand the importance of scheloribatid mites to the alkaloid defenses of poison frogs.

Although oribatids are a significant proximate source, the ultimate biological source of these alkaloids remains unknown. To more conclusively understand the origin of alkaloids in mites (and therefore in poison frogs), we propose three general research themes: (1) a series of feeding experiments in which cultured mites, raised through multiple generations, are provided a diet with and without alkaloids and/or precursors; (2) identification of the location of alkaloids within adult mites (e.g., are they located in the opisthonotal glands?); and (3) an attempt to culture the microbes (e.g., bacteria, fungi, etc.) present in mites in search for a possible alkaloid-producing microsymbiont.

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A Multi-Component Species Identifying Pheromone in the Goldfish

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Abstract Although it has been established that sexually-immature goldfish and their relatives recognize members of their own species by using chemicals that they release, the identity of this cue(s) and whether it might be produced and used by other life stages is not yet known. To address this question, this study tested the behavioral responses of sexually immature and mature goldfish to each other's body washings, their sensitivity to this cue, the role of the olfactory sense in detecting it, and whether it is comprised of either polar and/or non-polar compounds. Tests that used two-choice mazes discovered that juvenile, immature, mature male, and mature female goldfish all release and respond to a common chemical cue(s). Dilution studies next demonstrated that this cue is active when diluted over 10 times and thus capable of functioning as a short range attractant/identifier. Olfactory occlusion demonstrated that it is detected by the olfactory sense. Finally, chemical fractionation demonstrated that it is comprised of both polar and non-polar components but likely does not include bile acids. Together, these results suggest that all life stages of goldfish use a complex multicomponent pheromonal odor to discern species identity, and that this odor has the potential to function with hormonal metabolites to identify sexual condition in behaviorally active fish of many species.

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Introduction

Pheromones have been defined as chemical cues that convey information among individuals of the same species (Karlson and Lüscher, 1959; Wyatt, 2003, 2009). Cues that fit this definition are commonly used by teleost ('bony') fishes to mediate alarm, aggregation, and reproductive synchrony in the vast expanses of dark and/or turbid waters that they inhabit (Liley, 1982; Stacey et al., 1986; Stacey and Sorensen, 2009). All fish pheromones identified to date are body metabolite(s), a likely consequence of life in water (Sorensen and Hoye, 2010). Studies of the biological activity and identity of pheromonal odors in fish have taken two approaches. Several dozen studies have tested the actions of unpurified whole odors of immature and mature fish (body washings) and found that when tested, they generally elicit behavioral activity in a species-specific manner (Liley, 1982; Stacey et al., 1986; Baker and Montgomery, 2001; Sisler and Sorensen, 2008). Another set of studies has tested the activity of key hormonal metabolites ('hormonal pheromones') commonly produced by fishes, and found that they frequently stimulate olfactory and behavioral activity among closely related taxa (Stacey and Sorensen, 2005, 2009; Olsen et al., 2006). One possible explanation for these seemingly divergent results is that fish pheromones may function naturally as complex mixtures of metabolites, some of which identify species identity, while others (e.g., hormonal pheromones) change with maturity and time, thus adding information about physiological state.

The goldfish, *Carassius auratus*, is an excellent model to address whether and how a fish might use a species-

identifying pheromone because it is easily studied, well-understood, and seemingly typical of many other fishes (Stacey and Sorensen, 2009). Behavioral studies of immature (sexually-regressed) goldfish and the closely related common carp (*Cyprinus carpio*) show that these species both recognize their own species (Saglio and le Martret, 1982; le Martret and Saglio, 1982) and readily distinguish between each other and many other species by using waterborne chemicals that do not appear to be learned (Sisler and Sorensen, 2008). Further, a laboratory spawning study suggests that goldfish and carp do not readily hybridize (Sorensen et al., 1992) despite the fact that they use a common set of five hormonal compounds that mediate pre-spawning hormonal surges and reproductive behavior (Kobayashi et al., 2002; Stacey and Sorensen, 2009). Comparisons of the olfactory sensitivity and reproductive physiology of these species have yet to show any differences (Sorensen and Stacey, 2004). Intriguingly, a single study also has demonstrated that while male goldfish respond behaviorally to a metabolite of prostaglandin $F_{2\alpha}$ when it is added to goldfish holding water, males of another species (the blue gourami, *Trichogaster trichopterus*) do not become sexually aroused when the prostaglandin metabolite is added to their holding water (Sorensen et al., 2000, unpublished data). Although these data suggest that common goldfish body metabolites are perceived as a species-identifying cue, it has not yet been tested directly whether all life stages of any fish release a common subset of odorous compounds that might serve this function. This is an especially relevant question because physiological (reproductive) condition is known to modify many aspects of fish metabolism, sex hormonal pheromone release, and behavior (Stacey and Sorensen, 2009).

The chemical identity and biological significance of the compounds employed by goldfish to identify species also await resolution. Unique mixtures of bile acids may serve this role, as many fishes, including goldfish and related carps, are known to release and detect these relatively distinctive compounds with high sensitivity and specificity (Døving et al., 1980; Sorensen et al., 1987; Li and Sorensen, 1997; Derby and Sorensen, 2008). Complex mixtures of amino acids also could contribute because they too are released in quantity, and are detected with high sensitivity and specificity (Saglio and Blanc, 1989). Of course, other types of metabolites could also be involved. Notably, it has yet to be resolved whether the body washings (metabolites) of fishes are detected by the olfactory sense at concentrations low enough that they could function as pheromones in open waters.

The present study tested the possibility that all life stages of goldfish produce and perceive a common species-identifying pheromone by addressing five questions: 1) Does responsiveness of goldfish to conspecific washings remain constant with time as might be expected of a pheromone?; 2) Do all maturational stages of goldfish produce and respond to conspecific washings in the same manner?; 3) Are conspecific washings recognized by the sense of smell?; 4) Is the cue detected at low-enough concentrations for it to function as a pheromone?; and 5) Is this cue comprised of a single or multiple components, and might either bile acids and/or amino acids be involved?

Methods and Materials

Fish and Fish Care Goldfish were obtained from outdoor ponds (Hunting Creek Fish Farm, PA, USA) and shipped to the laboratory where they were held in 1000-l flow-through holding tanks supplied with well water (3-l/min; 17°C) on a 16:8 hr (L:D) photoperiod. Fish were fed a mixture of flake food (Aquatic Ecosystems, Apopka, FL, USA) and brine shrimp (San Francisco Bay Brand, CA, USA). Four groups of fish were used (Table 1). Briefly, juvenile fish were 3–6-mo-old and sexually undifferentiated. Sexually-immature fish were sexually regressed (previously mature) individuals that had been held in the laboratory for several years and had been previously used by Sisler and Sorensen (2008). Sexually-mature male and female fish were obtained from ponds several weeks prior to spawning, and were held in the laboratory as single sex groups to prevent them from stimulating each other (recent reproductive behavior or exposure to pheromones strongly enhances sexual receptivity and pheromone release; see Table 1 for more details on fish conditions). With the exception of the olfactory occlusion experiments, all fish were tested only once. Odor donors came from the same groups as the behavioral subjects.

Behavioral Assay Responses of goldfish were studied in the same circular mazes employed (and described) by Sisler and Sorensen (2008). Briefly, these mazes were 1.5 m diam and divided into two semi-circular sections that were connected by a 35 cm long central drained region. Each maze was illuminated by a set of dim (<1 lux) overhead incandescent bulbs and infrared light sources. A low-light overhead camera with a DVD recorder also was placed overhead. Well water (100 ml/min, 18°C) entered the central region of each maze and was maintained at a depth of 20 cm by using a drain. When appropriate (see below), odor stimuli were added to the semi-circular test sections through silicon tubes attached to air stones (to evoke mixing) using a peristaltic pump (10 ml/ min; Gilson, France). Control experiments have shown these mazes to be without bias (Sisler and Sorensen, 2008; Levesque, unpublished data). The day before each test, groups of 5 goldfish were moved into the mazes and allowed to acclimate.

Table 1 Characteristics of experimental fish

Life stages	Weight (g) N=4	GSI (%) N=4	Age	Time in laboratory	Notes
Juvenile	3.7±0.2	<0.42	3 mo	1 mo	Sexually undifferentiated
Immature	45.1±9.5	1.7±0.2	3–4 yr	3 yr	Regressed (nonvitellogenic oocytes, no sperm); Sexually non-responsive
Mature male	34.4±5.1	4.0±0.4	2 yr	3 mo	Presence of tubercles on pectoral fins and spermiated; Sexually responsive but not immediately receptive; Modest source of male sex pheromone[a]
Mature female	39.9±4.6	13.6±3.4	2 yr	3 mo	Distended abdomen with vitellogenic eggs; Sexually non-receptive; Modest source of steroidal pheromones[b]

Mean±Standard error

GSI = Gonadosomatic index (gonad weight /body weight body weight x 100)

[a] based on data from Sorensen et al. (2005)

[b] based on data from Scott and Sorensen (1994)

Experimental trials were conducted one at a time and started the next morning when the inflowing well water was turned off, and then 1 hr later, a 15-min pretest was conducted during which time blank well water (10 ml/min) was pumped into both sides of each maze. Pretests were followed by 15-min tests during which time either well water (control) or test odor (see below) were added to opposite sides of each maze while fish distribution was monitored. Test odor was added to the side that had been used least during the pretest to minimize possible bias. Trials concluded with a food odor control in which food odor (see below) was added to the least-used side for 15-min to confirm that fish were responsive to odors. All experiments were conducted 10 times with different groups of fish.

For data analysis, the numbers of fish on each side of each maze were counted at 15-sec intervals across each 15-min test period (pilot studies showed this to give a good representation of fish distribution). The total number of fish on each side was then summed for each test period and divided by the total number of times fish were seen on both sides after which the difference between the percent time fish spent on the test side during the pre-test period was subtracted from the percent time they spent during the test period to yield 'relative attraction'. Data then were summed for each experiment, evaluated for normality (Kolmogorov Smirnov test), transformed if appropriate (data for experiment 3 were arc-sin transformed), and then analyzed with one-way ANOVA (Instat, Graphpad Software, CA, USA). If significance was indicated (P<0.05), individual values were compared with the control in that experiment using Tukey Kramer *post-hoc* tests. In addition, well water and food odor controls within each experiment were compared to an expected value of zero using Student *t*-tests (Instat, Graphpad Software, CA) to ensure that there was no bias.

Test Odor Preparation Odor stimuli were prepared fresh each day following established protocols (Sisler and Sorensen, 2008). Briefly, approximately 100 g of goldfish (a value previously deemed to reflect that of a dense shoal of fish (Saglio and Blanc, 1989)) were carefully captured by hand net, weighed, and placed into clean plastic buckets containing 5- l of well water, and aerated by using a silicon air stone for 1-h. Blank control was prepared the same way without fish. Food odor was prepared by placing 1 g of fish food (tropical flake food, Aquatic Ecosystems Inc., Apopka, FL, USA) into 100 ml of well water for 1-h and then filtering it through filter paper (Grade 3,Whatman, International Ltd, Maidstone, England). Stimuli were used within 5 hr.

Experiment 1: Does Responsiveness of Juvenile Goldfish to Conspecific Washings Change with Time? This initial experiment sought to determine whether juvenile goldfish exhibit the same responses to conspecific washings as the sexually-immature goldfish that had been held for over a year in the laboratory. Not only did this question address whether this cue is a pheromone, but it allowed us to address whether we could use immature and juvenile fish interchangeably. Responses of both juvenile and regressed immature goldfish were tested to each other's body washings and blank water control in a randomized order. In a final test, juvenile goldfish were offered simultaneously the choice of juvenile and immature washings to confirm that they were equally attractive (thus reducing the possibility that fish may simply have appeared to have been equally attracted because these odors had been tested against a blank (a very weak competing stimulus).

Experiment 2: Do All Maturational Stages of Goldfish Produce and Respond to Conspecific Washings in the Same

Manner? Experiment 1 indicated that immature and juvenile goldfish release and respond to the same chemical stimuli; this experiment extended this approach to include sexually-mature males and females. All combinations of mature and juvenile fish and their body washings were tested. In addition, mature female goldfish were offered a choice of mature male vs. immature washings in one experiment, and mature male vs. mature female washings in another. The later experiments sought to confirm that these odors were perceived as being the same.

Experiment 3: Are Conspecific Body Washings Detected by the Olfactory Sense? To test the role of the olfactory sense in conspecific recognition, responses of immature goldfish were tested to body washing after their nares had been occluded, and then again after their occlusions had been removed. Immature fish were used because their large size made them easier to handle. For occlusion, fish were anesthetized in MS222® (tricaine methanesulfonate; 1 g/ 10 l), placed onto a wet paper, and an inert dental impression material (3M Express vinyl polysiloxane, St. Paul, MN, USA) was injected carefully into their nares following protocols previously shown not to affect fish swimming behavior or health (Maniak et al., 2000). Fish were tested with goldfish body washings within a day of occlusion, then anesthetized to remove their nasal occlusions using forceps, and given 3 week to recover before being re-tested.

Experiment 4: How Potent Is the Species-identifying Cue? Different groups of juvenile goldfish fish were tested to full strength body washings (20 g/ l), a 10-fold dilution of the former, and then a 100-fold dilution. The order of testing was randomized, and a well water control was included in the test matrix.

Experiment 5: Is this Cue Comprised of a Single or Multiple Components, and Might Either Bile and/or Amino Acids Be Involved? This experiment proceeded in two steps. The first tested whether the cue was polar and/or non-polar, while the second tested the role of bile acids found in the non-polar fraction. For the first step, 1-l aliquots of juvenile goldfish holding water (20 g/ l) were filtered (Whatman Grade 3, International Ltd, Maidstone, England), passed through activated reversed-phase C18 columns (Sep-pak Plus, Waters Corp., Milford, MA, USA), and eluted with 5 ml of methanol following established protocols (Polkinghorne et al., 2001). The eluate (i.e., the non-polar fraction) then was dried under a stream of nitrogen, reconstituted in well water to its original concentration, and its behavioral activity tested with juveniles. The filtrate (polar fraction) was tested directly in the maze. A mixture of the two then was tested to confirm that activity had not been lost. Pilot studies using C18 resins have shown that they do not release unknown compounds that influence fish behavior.

For the second step, the bile acid composition of goldfish holding water was examined following established protocols (Polkinghorne et al., 2001), and the bile acids found therein were tested for behavioral activity. Briefly, three 1-l aliquots of goldfish holding water were extracted as described above, dried, spiked with standards, injected onto a reversed-phase C18 column (Nova-pak C18, 4 μm column; Waters Corp., Milford, MA, USA) eluted with a gradient of acetonitrile and ammonium dihydrogen phosphate, and treated with 3α-hydroxysteroid dehydrogenase while fluorescence was monitored. Bile acids were identi-

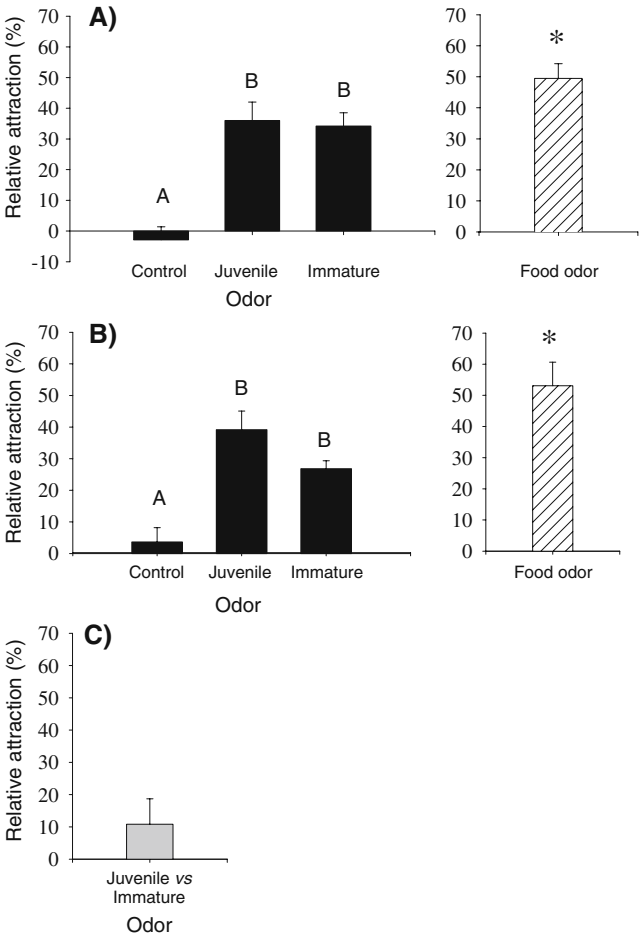


Fig. 1 **a** Changes in the distribution ('Relative attraction') of immature goldfish after the addition of either well water control, juvenile goldfish washings, or immature goldfish washings (mean percent change ± Standard error [SE]; N=10 groups of 5 fish in each case). Different letters denote statistical differences between treatments (P<0.05 by ANOVA). A summation of all food odor control experiments is shown to the right (*P<0.01 vs. 0 by paired t-test). **b** Relative attraction (mean percent change) of juvenile after addition of well water control, juvenile and immature goldfish washings (N=10). A summation of all food odor control experiments is shown to the right (*P<0.01 vs. 0.0 by paired t-test). **c** Changes in the relative distribution of immature goldfish offered a choice between juvenile and immature goldfish washings (N=10 groups of 5 fish)

fied based on retention times and mass spectrometry (Fine and Sorensen, 2008). The available compound (taurocholic acid) was purchased (Sigma Chemical Co, MO, USA), while the unavailable compound (cyprinol sulfate) was isolated and purified (99% purity by LC-MS) from goldfish gall bladders. These bile acids then were tested in our maze (in methanol carrier) at the concentration (10^{-10} Molar) estimated to be present in body washings.

Results

Experiment 1 found that while immature goldfish were not attracted to well water control ($P>0.10$), they were attracted to the body washings of both immature goldfish ($P<0.01$) and juvenile goldfish ($P<0.01$) (Fig. 1a). Similarly, juvenile goldfish were not attracted to well water ($P>0.10$) but were attracted to both washings of immature ($P<0.01$) and juvenile goldfish ($P<0.01$) (Fig. 1b). When juvenile goldfish were offered the choice of juvenile and immature body washings, they did not choose between them ($P>0.10$) (Fig. 1c). Food odor control was highly attractive to both groups ($P<0.01$) (Figs. 1a, b).

Experiment 2 demonstrated that all life stages of goldfish produced and discerned similar conspecific aromas. Juvenile goldfish were not influenced by well water control ($P>0.10$) but were highly attracted to washings of mature males ($P<0.01$) and mature females ($P<0.01$) (Fig. 2a). Mature females also were not attracted to the well water control but were attracted to washings of juveniles ($P<0.01$), mature females ($P<0.01$), and mature males ($P<0.01$). Further, when females were offered the choice of mature male vs. immature washings, and mature male vs. mature female washings, they showed no preference for either ($P>0.10$; Fig. 2c). Finally, mature males were attracted to the washings of juveniles ($P<0.01$), mature females ($P<0.01$) and mature males ($P<0.01$) (Fig. 2d).

Experiment 3 demonstrated that immature goldfish are attracted to conspecific washings because of their odor; they were not attracted to this stimuli when their nares were occluded ($P>0.10$), but they were attracted when occlusions were removed ($P<0.01$; Fig. 3). Experiment 4 showed this cue to be fully potent when diluted 10 times ($P<0.01$) but not 100 times (Fig. 4). The food odor control was attractive for all groups ($P<0.01$).

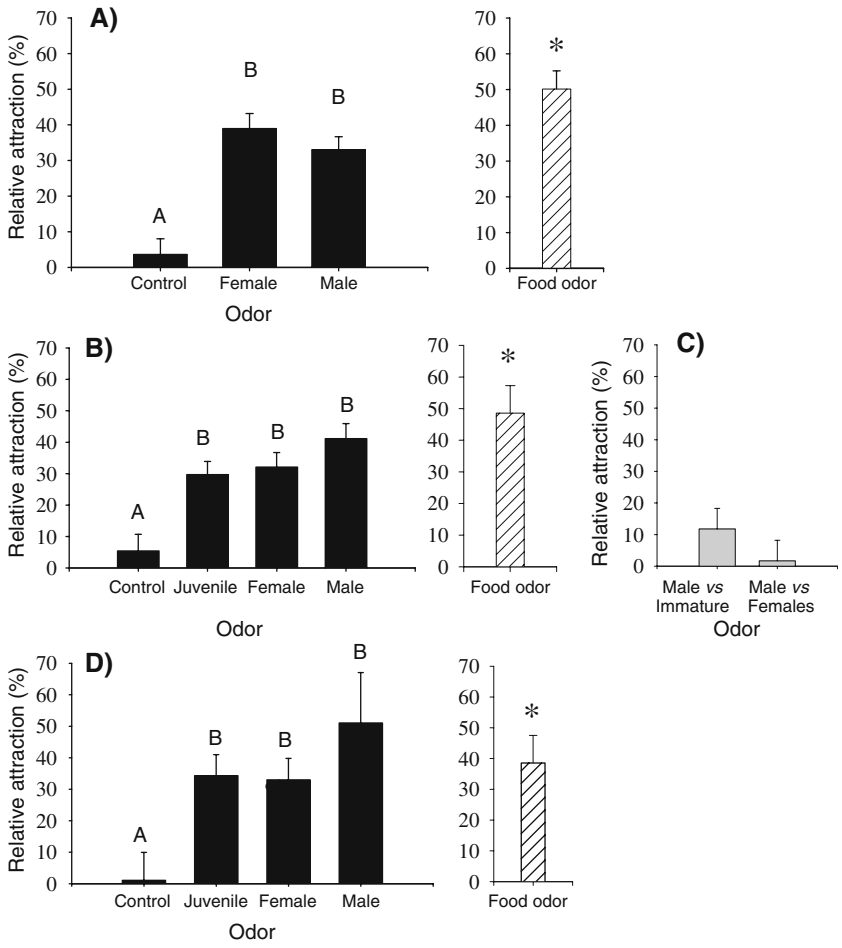


Fig. 2 **a** Relative attraction (mean percent change ± Standard error [SE]) of juvenile goldfish after the addition of either well water control, mature female, or mature male washings ($N=10$ groups of 5 fish; letters denote differences between treatments at $p<0.05$); A summated food odor experiment is shown to the right (*$P<0.01$ vs. 0.0 by paired t-test). **b** Relative attraction of mature female goldfish to well water control, juvenile, mature female, or mature male goldfish washings ($N=10$; $P<0.05$). The summated food odor control experiment is shown to the right (*$P<0.01$); **c** Changes in the relative distribution of mature female goldfish offered a choice between mature male and immature goldfish washings, and between mature male and mature female washings ($N=10$ for each; $P<0.05$); **d** Relative attraction of mature male goldfish to well water control, juvenile, mature females, or mature male washings ($N=10$ groups of 5, $P<0.05$). The food odor control is shown to the right (*$P<0.05$)

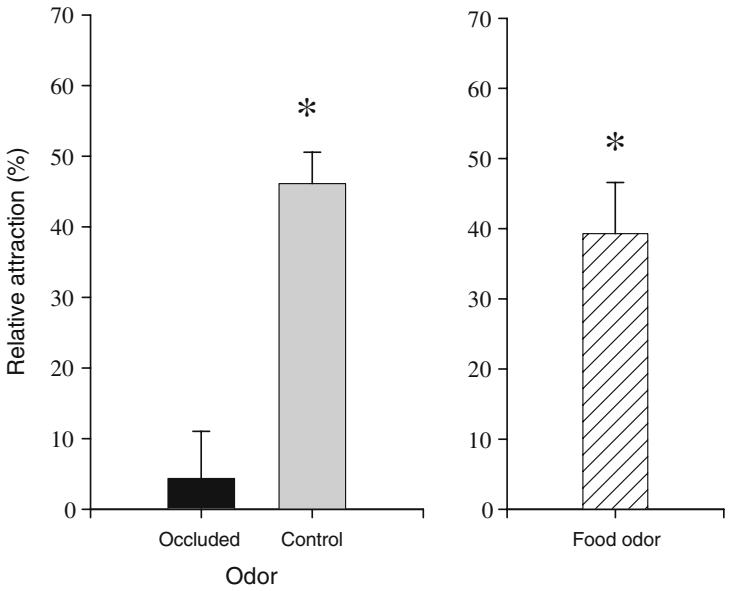


Fig. 3 Changes (mean percent change ± Standard error [SE]) in the relative distribution of immature goldfish with occluded nostrils (black bar) and the same fish after their occlusions had been removed (clear bar) to immature goldfish body washings (N=5 groups of 10; *P< 0.01 vs. 0 for both treatments). The food odor control for the post-occluded group (*P<0.05 vs. 0) is shown to the right

Finally, experiment 5 showed that both the non-polar C18 eluate and the polar filtrate of juvenile holding water were attractive to juvenile goldfish (P<0.05; Fig. 5). A combination of the eluate and filtrate had potency equivalent to untreated juvenile holding water (P>0.10), and also was more attractive than either the filtrate or eluate alone (P<0.05; Fig. 5). HPLC analysis of goldfish water found large quantities of two identifiable bile acids, cyprinol sulfate (CS) and taurocholic acid (TCA) (Fig. 6a). The former was released at a rate of 4.2 μg / l / 20 g body weight of goldfish for CS and the later at a rate of 0.085 μg / l / 20 g. A mixture of these bile acids had no measurable effect on the behavior of juvenile goldfish (P>0.10; Fig. 6b).

Discussion

This study confirms the existence of a species-identifying cue in immature goldfish body washings (Saglio and le Martret, 1982; Sisler and Sorensen, 2008), while demonstrating that juveniles, mature males, and mature females all produce and respond to a similar conspecific cue. The fact that neither juvenile nor female goldfish distinguished between mature or juvenile fish washings in head-to-head behavioral tests, argues strongly that maturity does not influence the production or discrimination of this cue, whose specificity has already been described in some detail by Sisler and Sorensen (2008). Because the species-identifying cue is active at low concentrations and it is detected by the olfaction, it should be considered a pheromone. Fractionation suggests that it has multiple non-polar and polar components. This appears to be the first systematic description of the ability of a fish species to identify all life history stages of conspecifics by using odor alone. Previous work allows us to apply this finding to the closely-related common carp (Sisler and Sorensen, 2008).

This species-identifying pheromone should be adaptive to the goldfish and its relatives that live in turbid waters, and forage and spawn in groups (McCrimmon, 1968). Our dilution experiments suggest that the primary role of the pheromone may be as a close range identifier for shoaling and spawning. Although the present study did not examine the full range or complexity of pheromonal compounds released by goldfish (the specific role of sexual cues was not examined), it nevertheless does demonstrate that different life history stages consistently release common compounds that are consistently perceived as a distinctive species-specific entity by all life stages. The presence of a species-specific odor could explain previous observations that goldfish and carp do not readily hybridize despite the fact that

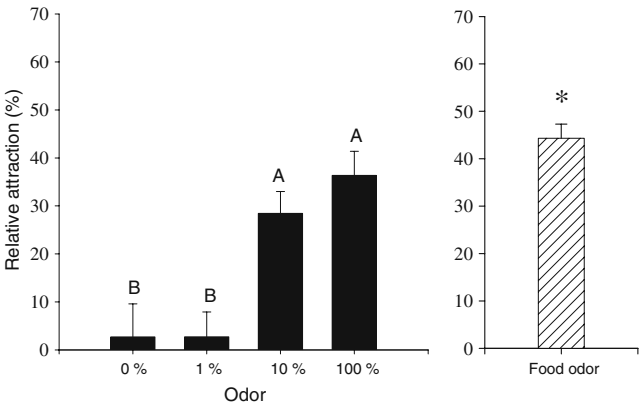


Fig. 4 Relative attraction (mean percent change ± Standard error [SE]) of juvenile goldfish to well water control, a 1% dilution of juvenile washings, 10% juvenile washings, juvenile odor (100%) odor, and then food control (N=10 groups of 5; different letters indicate differences; P<0.01)

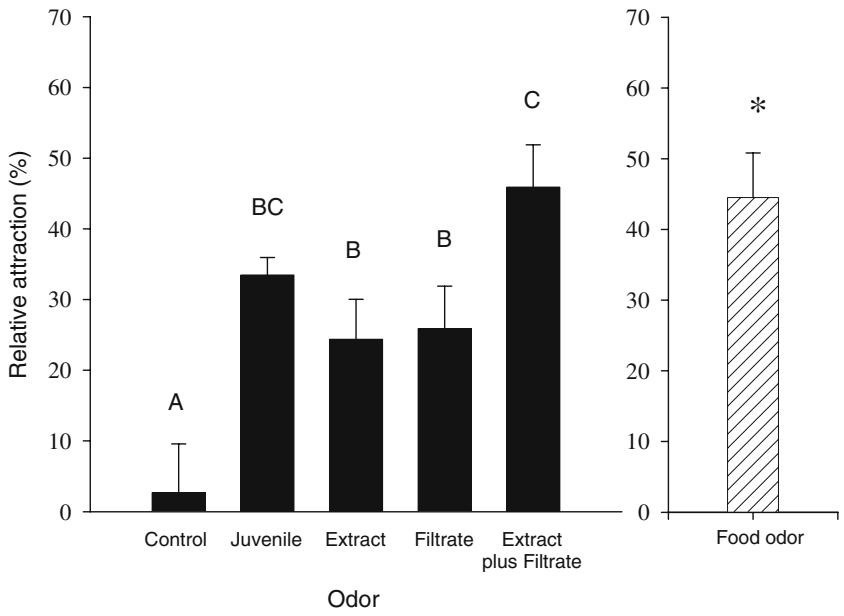


Fig. 5 Relative attraction (mean percent change ± Standard error [SE]) of juvenile goldfish after the addition of well water, juvenile goldfish, juvenile goldfish C18 extract, C18 filtrate, or a mixture of C18 extract and filtrate [N=10 groups of 5; significant differences between treatments (P<0.05) noted by letters]. The summated food control is noted to the right (*P<0.05 vs. 0)

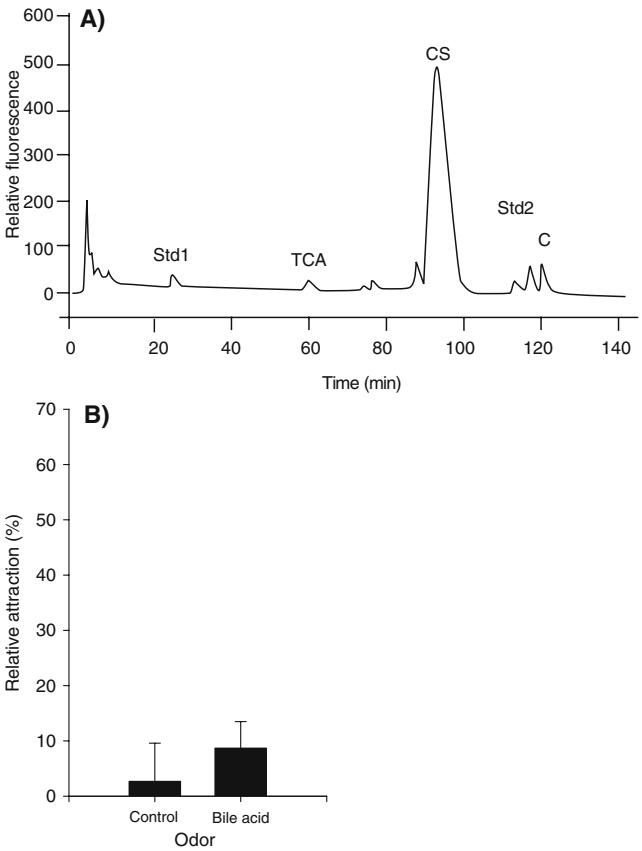


Fig. 6 **a** A representative chromatogram showing the bile acids found in goldfish holding water. Y-axis shows relative fluorescence and x-axis time. CS, cyprinol sulfate; TCA, Taurocholic acid; Std1, standard 1 (0.1 μg hyocholic acid); Std2, standard 2 (0.1 μg lithocholic acid); c. unknown non-bile acid contaminant. **b** Changes (mean percent change ± Standard error [SE]) in the relative distribution of juvenile goldfish exposed to either well water control or a mixture of CS and TCA (no differences when compared to each other by t-test)

they share the same hormonal pheromones (Sorensen et al., 1992), and that altering body odor background could alter the function of hormonal >pheromones (Sorensen et al., 2000; Sorensen and Levesque, unpublished data). If true, this phenomenon would answer the paradox of how various fish species can employ common hormonal products as species-specific sex pheromones (Sorensen and Stacey, 1999; Stacey and Sorensen, 2005, 2009). A similar scenario can be envisaged for the fish alarm cue, which some suggest to be a simple purine (Brown et al., 2000), but which detailed cross-species comparisons using whole odors often show to be species-specific (Smith, 1992). The sophisticated neural architecture of the fish olfactory system appears capable of discriminating odor complexes (Friedrich and Korsching, 1998; Michel, 2006; Derby and Sorensen, 2008). Further study of pheromone mixtures would be timely.

The present study demonstrates that the species-identifying pheromone recognized by the goldfish is comprised of both non-polar and polar components, and all components need to be present for full activity. Somewhat surprisingly, the two bile acids we isolated from goldfish waters, cyprinol sulfate (a bile acid novel to the cyprinid minnows; Tammar, 1974) and taurocholic acid, seemed unable to explain the biological activity of the non-polar fraction although both are detected by the goldfish with picomolar sensitivity (Sorensen et al., 1987; Sorensen and Stacey, 2004; Sorensen, unpublished data). However, while bile acids and related compounds have been shown to exhibit pheromonal activity in an ancient cartilaginous fish, the sea lamprey (*Petromyzon marinus;* Li et al., 2002; Fine and Sorensen, 2008), their roles as behavioral stimuli have to date been equivocal in bony fishes (Baker et al., 2006). More detailed tests are needed. Although our study did not

address the chemical identity of the active component(s) in the polar fraction, it remains possible that it is comprised of mixtures of amino acids as suggested by Saglio and Blanc (1989). Future work should fully fractionate goldfish and carp body washings to determine the identity of these pheromones.

In summary, this study has demonstrated that all life stages of goldfish produce and use a common species-specific pheromone. The pheromone awaits identification although pilot data suggest that bile acids may not play a prominent role. Because the goldfish has proven to be a good model for many fish, it seems likely that that other fish species use similar cues. It will be especially interesting to test directly how this species-identifying pheromone influences perception and function of hormonal pheromones.

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Letter from the Editorial Board

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New Synthesis. Thirty five years have passed since the Journal of Chemical Ecology (JCE) was first published. A glance through the contents of that inaugural volume reveals the zeitgeist of our field in 1975: papers on pheromone identification, defensive secretions, chemical attraction (or repellence), and the methods required to study these subjects, primarily by using insects and terrestrial plants as model systems. Although these themes remain current, recent issues of JCE have featured studies of cuticular hydrocarbons (especially in social insects), tri-trophic interactions, phenotypic plasticity, metabolomics, genomics and other informatics approaches, with a growing number of such studies using marine and/or microbial systems. These changes are symptomatic of a genomics-age "new synthesis" in the biological sciences, in which researchers working at levels of organization as different as coral reefs and insect guts increasingly use similar analytical tools to understand how complex interactions mediate the generation and maintenance of biological diversity.

Here, we initiate a new feature for JCE, entitled "New Synthesis", in which short essays will highlight exciting new conceptual and/or technological trends in Chemical Ecology. Chemical Ecology has always played a prominent role in the development and testing of theory in the fields of sexual selection, plant-herbivore interactions and integrated pest management. In "New Synthesis", we will trumpet the growing impact of Chemical Ecology on the study of symbiosis and mutualism, molecular ecology, community genetics and ecosystem-level biogeochemistry, among other fields.

Our goal is for these "essays" to appear monthly and to obtain contributions from important labs worldwide. Their appearance in all likelihood will be somewhat sporadic at first. For now, we are targeting individuals who have expressed interest and who are willing to write. We hope for a variety of perspectives. The thoughts of both innovators and those with mature vantage points are desired. These contributions will be subject to editorial but not peer review. We envision them to focus on a single or small group of papers and to be no longer than 500 words. We hope this will become a useful feature, and we encourage your participation.

Robert A. Raguso, Editorial Board Member and John T. Romeo, Editor-in-Chief

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COMMENTARY

New Synthesis—Trade-offs in Chemical Ecology

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Although many chemical ecologists may not consider themselves evolutionary biologists first and foremost, evolutionary thinking pervades much of our science. Indeed, the concept of trade-offs is central to nearly all arguments about adaptation—what else would limit organisms from being perfectly adapted to all predators, changing climates, and sundry other life challenges? And such trade-offs (aka costs) often are held as a holy grail for those trying to explain variation in investment in traits, including chemical compounds, assumed to be adaptive. Nonetheless, we still lack a taxonomy for the causes and types of trade-offs. Trade-offs in chemical ecology may be caused by resource limitation, direct chemical interference, suppression of genes or biosynthetic pathways, or even divergent natural selection on a single trait. New papers, discussed below, contribute to the discourse working towards this needed taxonomy.

Because chemical compounds intertwine within organisms and biosynthetic pathways intersect during expression, there is great potential for trade-offs in chemical ecology. Recently, Ballhorn (2011) and Ballhorn et al. (2010) demonstrated the *direct chemical interference* of one plant defense by another and its ecological consequence. Using cultivated and wild lima bean (*Phaseolus lunatus*), they first report a pattern: insect resistant, high cyanide (HCN) bean accessions were susceptible to a fungal pathogen. Indeed, this pattern also was reported for clover by Rodolfo Dirzo in the early 1980s. But what is the mechanism? Ballhorn et al. (2010) show that HCN directly disrupts the enzymatic activity of polyphenol oxidase (PPO), a likely mechanism of resistance to fungi. Thus, a direct chemical trade-off results in a dilemma for plants: to resist insects or pathogens? A similar ecological pattern could, however, occur via an *ecological trade-off*. In this case, a single compound may be deterrent to some herbivores and attractive to others, as has been well-documented for cucurbitacins and some glucosinolates.

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Of course, historically, and even predating Darwin, *resource-based trade-offs* were presumed to be the rule—that is, resources allocated to one trait simply cannot be allocated to another trait. Although resource-limitation clearly is important at the level of the whole organism, resource-based trade-offs are unlikely to be the direct drivers of most negative correlations between two traits. In fact, we realize now that some negative associations between traits may be *adaptive negative correlations*. Take, for example, the oft cited trade-off between chemical resistance and tolerance. Tolerance is the ability of plants to withstand and regrow following herbivory. Theory and some evidence has suggested that highly resistant plants, need not tolerate damage (and *vice versa*, highly tolerant plants do not experience selection for resistance to herbivory). If these traits are, indeed, ecologically redundant and even a little energetically costly, then a "trade-off" may occur because selection has favored it. Current mechanistic work is showing how tolerance mechanisms can be induced following herbivory. Nonetheless, we know little about the mechanistic basis of variation in tolerance, and its relation to chemical resistance among plant genotypes or species.

Finally, neither the mechanistic nor evolutionary basis of some trade-offs may be known, even if quite general. *Negative hormonal cross-talk* between jasmonate and salicylate in plant defense signaling appears to be nearly ubiquitous. Is this a resource-based trade-off? Or does it make sense for plants—when one is employed the other is not needed? It seems unlikely to be direct chemical interference. Whatever the case, all sorts of organisms manipulate these pathways to their benefit and perhaps to the decrement of plants. The latest case is the induction of salicylate by insect eggs, which reduces jasmonate-induction following egg hatch, and thereby benefits caterpillars (Bruessow et al., 2010). Isn't chemical ecology wonderful!

But remember, nearly all trade-offs can be overcome by natural selection—they just have to be important enough. Thus, an important next step is understanding the evolutionary context of trade-offs among compounds that mediate ecological interactions.

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Release of Hydrogen Cyanide via a Post-secretion Schotten-Baumann Reaction in Defensive Fluids of Polydesmoid Millipedes

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Abstract Mandelonitrile benzoate, a minor defense component produced by polydesmoid millipedes, is produced in large amounts together with hydrogen cyanide following shake-disturbances administered to individuals of *Nedyopus tambanus tambanus*, *Parafontaria tonominea*, *Epanerchodus* sp., and *Epanerchodus fulvus.* These species commonly produce mandelonitrile and benzoyl cyanide (the oxidized product after discharge). The newly generated mandelonitrile benzoate was identified as a product of post secretion Schotten-Baumann reaction under basic conditions of bled bodily fluids (pH ca. 9.0), and was not an enzymatic reaction product. The reaction occurred *in vitro* even under less basic conditions [1*M* Tris-HCl buffer (pH 8.0)], and could be defined as a new mechanism of hydrogen cyanide release occurring in roughly half of polydesmoid millipedes. Species possessing no benzoyl cyanide, such as *Oxidus gracilis* and *Cryptocorypha* sp., could also produce mandelonitrile benzoate under conditions in which benzoyl cyanide was exogenously provided.

Key Words *Nedyopus tambanus tambanus* · Mandelonitrile benzoate · Mandelonitrile · Benzoyl cyanide · Defense secretion · Polydesmida · Schotten-Baumann reaction

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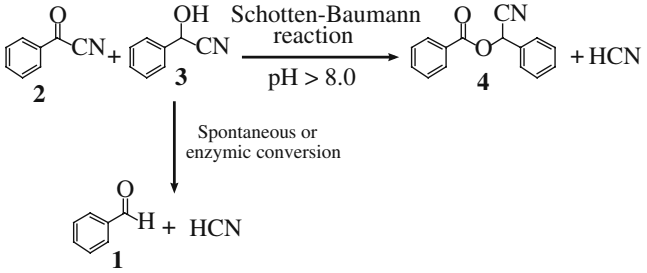
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Introduction

Hydrogen cyanide is a toxic component widely distributed by arthropods and plants. It is believed to function as a defensive agent against predators or as a toxin to herbivores (Zagrobeiny et al., 2008). With the exception of a few non-cyanogenic Polydesmida such as *Niponia nodulosa* Verhoeff (Ômura et al., 2002a), *Eutrichodesmus elegans* Miyosi (Kuwahara et al., 2002), and *Eutrichodesmus armatus* Miyosi (Kuwahara et al., 2003), the majority of polydesmoid millipedes are cyanogenic, and generally are identified as containing mandelonitrile {**3**, benzaldehyde (**1**) cyanohydrine} (Mori et al., 1994; Kuwahara, 1999). Benzoyl cyanide (**2**) also is recognized as one of the major defense components of three polydesmoid millipedes (Conner et al., 1977) and of twelve species belonging to Xystodesmidae (Duffey et al., 1977).

The major constituents in the hexane rinse of *Nedyopus tambanus tambanus* are a mixture of **2** and **3**, whereas its gland's reservoir has been shown to only contain **3** (Kuwahara et al., 2008). To obtain substantial amounts of endogenous **3**, a live millipede contained in a glass vial was shaken vigorously (shake-disturbance) and then extracted by hexane. Unexpectedly, the resulting extracts contained large amounts of mandelonitrile benzoate (**4**, reaction product of **2** and **3**). The formation of **4** as a major component also was demonstrated from shake-disturbance in the three other Polydesmida.

In this study, the unusual mandelonitrile benzoate (**4**) formation was identified as the result of a Schotten-Baumann reaction between **2** and **3** (Scheme 1); a new mechanism that produces rapid emission of hydrogen cyanide. The distribution of this reaction also is discussed among related polydesmoid millipedes together with their defensive functions.



Scheme 1 Hydrogen cyanide release mechanisms evidenced in cyanogenic polydesmoid millipedes

Methods and Materials

Millipede Species The following six millipedes were used: *Nedyopus tambanus tambanus* (Attem) 1901 (Polydesmida: Paradoxomatidae); *Parafontaria tonominea* Attems, 1899 (Polydesmida: Xystodesmidae); *Epanerchodus fulvus* Haga 1956 (Polydesmida: Polydesmidae); *Oxidus gracilis* C. L. Koch (Polydesmida: Paradoxosomatiae); *Cryptocorypha* sp. (Polydesmida: Pyrgodesmidae); and *Epanerchodus* sp. (Polydesmida: Polydesmidae). *Nedyopus tambanus tambanus*, *P. tonominea* and *Ep. fulvus* were collected around the campus of Kyoto Gakuen University in Kameoka in the Kyoto Prefecture. *Cryptocorypha* sp. was obtained from litters in the Osaka prefecture, as well as on the campus of Kyoto Gakuen University. *Oxidus gracilis* was collected from litters in Kyoto city, and *Epanerchodus* sp. (at the 7th stage, examined by T. Tanabe) from litters at Lake Usui in the Gunma Prefecture. Individuals were maintained by feeding rotten plant material. Specimens of unidentified millipedes, *Cryptocorypha* sp. and *Epanerchodus* sp., were deposited in the Kumamoto University (Kumamoto Japan 860-8555) millipede collections (T. Tanabe, accession number D5120 and D5121, respectively).

Chemicals Mandelonitrile (**3**, tech. grade), benzoyl cyanide (**2**, 98%), and n-tetradecane (99+%) were obtained from Sigma-Aldrich Co, Japan. Benzaldehyde (**1**, reagent grade, Wako Pure Chem. Co. Ltd. Japan), n-hexane (reagent grade, Wako Pure Chem. Co. Ltd. Japan), and others were commercially available. Mandelonitrile benzoate (**4**) was prepared by a Schotten-Baumann reaction as described below.

Chemical Analysis Gas chromatography-mass spectrometry (GC-MS) analysis was conducted using a Network GC System (6890N; Agilent Technologies Inc.) coupled with a mass selective detector (5975 Inert XL; Agilent Technologies Inc.) operated at 70 eV, using an HP-5MS capillary column (0.25 mm i.d.×29 m, 0.25 μm film thickness; Agilent Technologies Inc.). Helium was used as the carrier gas at a flow rate of 1.00 ml/min, with a split-less mode at a temperature programmed to change from 60°C (2 min) to 290°C at a rate of 10°C/min. This temperature was maintained for 5 min. GC and GC-MS data were obtained by Chemstation (Hewlett Packard Co.), with reference to an MS database (Wiley275 library; Hewlett Packard Co.).

pH of Millipede Body Fluids The pH of two species, *Ne. tambanus tambanus* and *O. gracilis* were measured by pH indicator paper (pH 1–14; Johnson Test Papers Ltd.).

Detection of Hydrogen Cyanide A piece of sodium picrate paper was exposed to the atmosphere of a test vial containing a millipede to determine the presence of hydrogen cyanide.

Conventional Hexane Extraction of Millipedes (Total Six Species) Each adult (or immature) millipede was submerged alive in a screw-capped glass vial (10 ml, 5 ml, or 1 ml in volume, depending upon the body size of the millipede species) that contained the following amounts of hexane; 0.5 ml for *Ne. tambanus tambanus*; 2 ml for *P. tonominea*; 0.3 ml for *Epanerchodus* sp.; 0.1 ml for *Ep. fulvus*; 1 ml for *O. gracilis*; and 0.05 ml for *Cryptocorypha* sp. The resulting hexane extracts, collected by decantation, then were analyzed by GC-MS.

Hexane Extraction of Shake-disturbed Millipedes (Total Six Species) Before extraction, each live millipede was shaken vigorously in a glass vial by hand for 2 min. Hexane (volume for each species, as above) was then added, and vials were kept for 3 min at room temperature. Secretions smeared on the inner wall of the vial were simultaneously extracted and recovered. The extract was collected by decantation followed by GC-MS analysis.

*Hexane Extraction of Shake-disturbed Millipedes Kept in Vials with Exogenously Provided Benzoyl Cyanide (**2**) (Total Two Species)* The following procedure was applied to the species *O. gracilis* and *Cryptocorypha* sp. Either 100 μg (*O. gracilis*) or 30 μg (*Cryptocorypha* sp.) of authentic benzoyl cyanide (**2**, dissolved in hexane) was exogenously introduced into each vial. The millipede then was added and shake-disturbed following evaporation of the solvent. The millipede and inner wall of the vial were extracted for 3 min by hexane as above, and extracts then were analyzed by GC-MS as described.

*Incubation of **2** and **3** Mixtures with Macerated Millipede* To explore the possibility of an enzymatic reaction, a macerated *Ne. tambanus tambanus* (as the "crude enzyme") was incubated in hexane (0.1 ml) containing a mixture of **2** (100 μg) and **3** (100 μg), together with n-tetradecane (100 μg) as an internal standard. The "crude enzyme" was prepared by crushing a millipede with a glass rod, followed by subsequent washing (twice) with hexane to remove non-

polar components and any secreted defense compounds. Following incubation, organic layers were analyzed by GC-MS at intervals of 3, 40, and 85 min, respectively, to monitor reaction during the hexane incubation period. As a control, a macerated millipede body subjected to a treatment of 100°C for 30 min in a small, sealed glass tube (heat denatured) was incubated similarly at room temperature.

Schotten-Baumann Reaction Between **2** *and* **3** *Using Tris-HCl Buffer (pH 8.0)* A neat stock mixture was prepared by dissolving compounds **2** (131 mg, 1 mmol) into compound **3** (133 mg, 1 mmol) without a solvent. A drop of 1 *M* Tris-HCl buffer (pH 8.0) was added to a portion of the stock mixture (**2** and **3**, less than 10 mg) in a test tube (5 cm length × 0.7 cm o.d.). After 1 min, the reaction mixture was extracted with hexane. After appropriate dilution, this extract was analyzed by GC-MS.

As a control, one drop of distilled water was similarly added to the stock (**2** and **3**, less than 10 mg). After 1 min, the resulting mixture was extracted similarly, diluted by hexane, and analyzed by GC-MS.

Results and Discussion

The defense secretion of *Ne. tambanus tambanus* was directly sampled by using a glass capillary and was found to contain solely mandelonitrile **(3)**, whereas the conventional solvent extracts contained a mixture of benzoyl cyanide (**2**, 23.1±9.5 μg in males and 13.8±2.7 μg in females) and mandelonitrile (**3**, 34.6±5.6 μg in males and 30.9±5.6 μg in females) (Kuwahara et al., 2008). The content of **3** was calculated from that of benzaldehyde (**1**, a thermally decomposed product from **3** during GC-MS analysis, Mori et al., 1995).

As discussed earlier, endogenous **3** was not obtained by shaking (shake-disturbance) and subsequent hexane rinse. The GC profile of the hexane extracts, unexpectedly, showed a major peak at t_R 18.18 min for mandelonitrile benzoate (**4**) as the most abundant component (Fig. 1b), together with two small peaks at t_R 5.67 min (artifact from **3**) and 7.80 min for **1** and **2**, respectively. Although the ester (**4**) is commonly distributed among Polydesmoid millipedes as a minor component (Duffey et al., 1977; Eisner et al., 1978), the shake-disturbance of *Ne. tambanus tambanus* increased its production by 50 fold, although **2** and **1** (artifact from **3**) decreased, respectively, to 1/10 and 1/4 of the control (undisturbed and conventional solvent extraction) (Fig. 1a). Hydrogen cyanide release upon shake-disturbance also was confirmed by the development of dark orange color on sodium picrate paper. During shaking, the inner surface of the vial became wet from smeared defense secretions and/or bodily fluids that bled out.

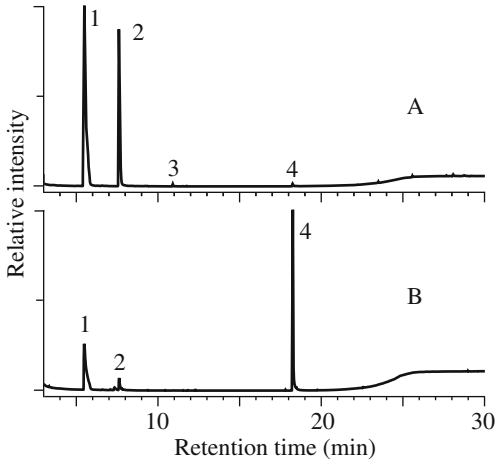


Fig. 1 A typical GLC of the defense secretion from *Nedyopus tambanus tambanus*. **a** Conventional hexane extracts; **b** Shake-disturbed extracts. **1**, benzaldehyde; **2**, benzoyl cyanide; **3**, mandelonitrile; and **4**, mandelonitrile benzoate

This effect of shake-disturbance similarly was examined in *O. gracilis*, whose defense secretions consisted only of **3** (detected as **1** by GC) as cyanogen together with non-cyanogenic minor components (Taira et al., 2003). No difference was observed between GC chromatograms of the conventional extract (Fig. 2a) and extracts post

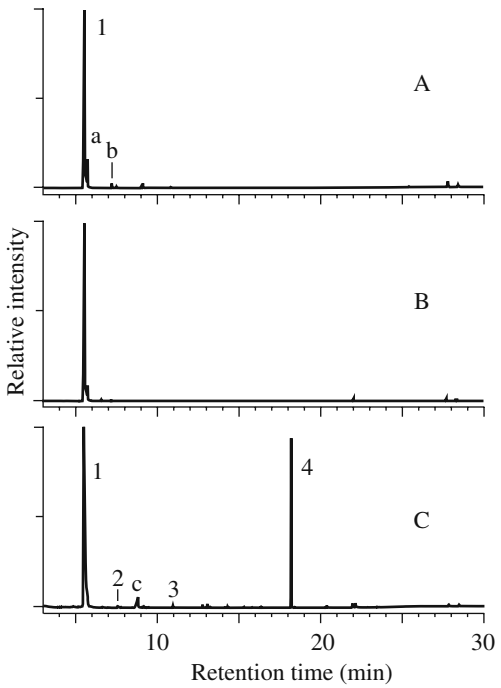


Fig. 2 A typical GLC of the defense secretion from *Oxidus gracilis*. **a** Conventional hexane extracts; **b** Shake-disturbed extracts; **c** Shake-disturbed extracts upon addition of benzoyl cyanide. **1**, benzaldehyde; **2**, benzoyl cyanide; **3**, mandelonitrile; **4**, mandelonitrile benzoate; a, phenol; b, *p*-cresol; and c, benzoic acid

shake-disturbance (Fig. 2b), while shake-disturbance with exogenously introduced **2** (100 μg) produced **4**, as summarized in Fig. 2c. Based on these observations, it is clear that the production of **4** is dependent on the occurrence of **2** and **3**.

Among eleven Japanese cyanogenic Polydesmida (Table 1), the following six species contained **1** (as **3**), **2**, and **4** together with sporadic detection of **3** by GC: *Ne. tambanus tambanus* (Kuwahara et al., 2008), *P. tonominea* (Ômura et al., 2002b), *Epanerchodes sp*., *Ep. fulvus*, *Chamberlinius hauliensis* (Noguchi et al., 1997a), and *P. laminata armigera* (Mori et al., 1995). In addition to *Ne. tambanus tambanus*, the following three species demonstrated the effect of shake-disturbance to produce large amounts of **4**, as summarized in Fig. 3: *P. tonominea*, *Epanerchodes* sp., and *Ep. fulvus*. In the case of *P. tonominea*, **4** increased 21 fold, with a decrease of **2** to 1/46 and a 1.6 fold increase of **1**. In the case of *Epanerchodus* sp., **4** increased 94 fold, with a decrease of **1** and **2** to 3/16 and 17/48, respectively. In the case of *Ep. fulvus*, **4** increased sevenfold, with an unchanged level of **2** and a decrease of **1** to 3/19. Shake-disturbance, therefore, promoted significant production of **4**, with a corresponding decrease in production of **1** and **2**.

In contrast, the following five species release compound **3** (detected as **1** by GC) as only one cyanogen together with non-cyanogenic minor components: *O. gracilis* (Taira et al., 2003), *Cryptocorypha* sp., *Ep. japonicus* Carl. (Mori et al., 1994), *Riukiaria semicircularis semicircularis* (Ômura et al., 2002b), and *Ne. patrioticus patrioticus* (Noguchi et al., 1997b). Neither **2** nor **4** were detected in these species, except in cases of exogenously introduced **2** (100 μg) to *O. gracilis*, as described earlier. The same shake-disturbance effects were reproduced in *Cryptocorypha* sp. in the presence of exogenously introduced **2** (30 μg), as summarized in Fig. 3 and Table 1.

The ester (**4**) formation was hypothesized to occur through the process of enzyme(s) reaction; the possibility of this process was explored with *Ne. tambanus tambanus*. An entire hexane-washed millipede body submerged in the substrate (hexane solution of **2** and **3**) did not generate **4**, although a macerated body catalyzed the production of **4**. However, even after heat treatment, the reactivity to **4** did not decrease, and **4** was similarly produced during 85 min incubation at room temperature together with decreased production of **2** and **3** (as **1**) (Fig. 4). Therefore, enzyme(s) reaction is excluded as a possibility for the demonstrated formation of **4**.

Table 1 Detection of mandelonitrile benzoate as a component of defense secretion among Japanese Polydesmida

Species	Family	Solvent	Bz-al (**1**)	BzCN (**2**)	MN (**3**)	MDBZ (**4**)	*ϕ-OH*	*p*-Cr	Bz-OH	Bz-O-Me	Ref.
Nedyopus tambanus tambanus	Paradoxosomatidae	Hexane	+	+	+	+(+) Fig. 1					1)
Parafontaria tonominea	Xystodesmidae	Hexane	+	+	+	+(+)					2)
Epanerchodus sp.	Polydesmidae	Hexane	+	+		+(+)					
Epanerchodus fulvus Haga	Polydesmidae	Hexane	+	+	+	+(+)					
Chamberlinius hualienensis[a]	Paradoxosomatidae	MeOH	+	+		+			+	+	3)
Parafontaria laminata armigera[a]	Xystodesmidae	CH_2Cl_2	+	+		+			+		4)
Oxidus gracilis	Paradoxosomatidae	MeOH	+			(+) Fig. 2		+	+	+	5)
Cryptocorypha sp.	Pyrgodesmidae	Hexane	+		+	(+)					
Epanerchodus japonicus Carl[a]	Polydesmidae	CH_2Cl_2	+				+				6)
Riukiaria semicircularis semicircularis[a]	Xystodesmidae	Hexane	+								2)
Nedyopus patrioticus patrioticus[a]	Paradoxosomatidae	MeOH	+				+	+			7)

Bz-al: benzaldehyde, BzCN: benzoyl cyanide, MN: mandelonitrile, MDBZ manndelonitrile benzoate, *ϕ*-OH: phenol, *p*-Cr: p-cresol, Bz-OH: benzoic acid, and Bz-O-Me: methyl benzoate.

(+): A detectable amount of manndelonitrile benzoate (**4**) was produced, when a millipede was shake-disturbed with added benzoyl cyanide (**2**). See the text

+(+): Shake-disturbance enhanced the production, though originally detectable in the extract. See the text

[a] Species in which shake-disturbance experiment has not yet been tried

1) Kuwahara et al., 2008, 2) Ômura et al., 2002b, 3) Noguchi et al., 1997a, 4) Mori et al., 1995, 5) Taira et al., 2003, 6) Mori et al., 1994, 7) Noguchi et al., 1997b

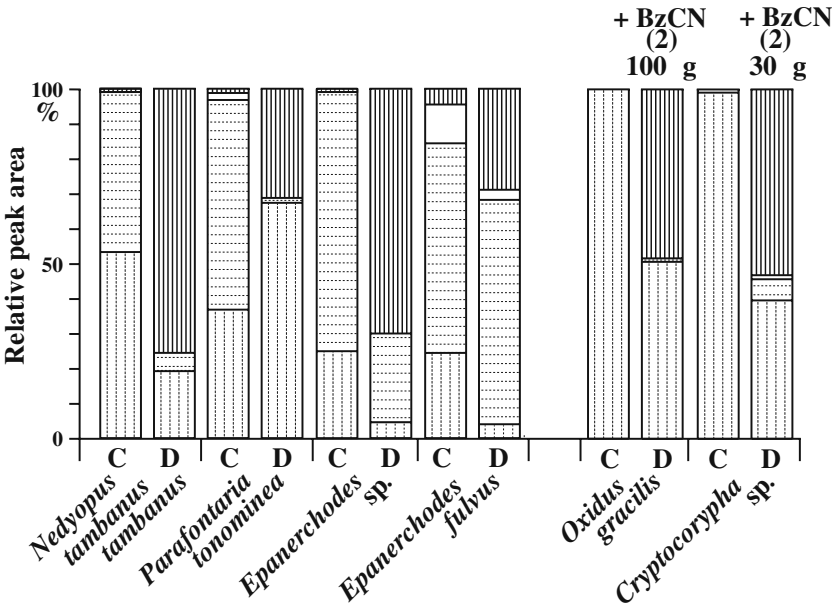


Fig. 3 Relative changes in the composition of defense secretions, based on relative peak heights between control (C) and shake-disturbed (D) on individual from six millipede species.
: benzaldehyde (**1**),
: benzoyl cyanide (**2**),
: mandelonitrile (**3**),
: mandelonitrile benzoate (**4**)

This result, although puzzling at first, led us to discover a new mechanism of hydrogen cyanide release (cyanogenesis) distributed among cyanogenic millipedes. Based on the following evidence, formation of **4** was inferred as the Schotten-Baumann reaction (Kuerti and Czako, 2005) between **2** and **3** under alkaline body fluids conditions: 1) the reaction occurs in the presence of body fluids that may bleed from shake-disturbance; 2) the body fluids are basic

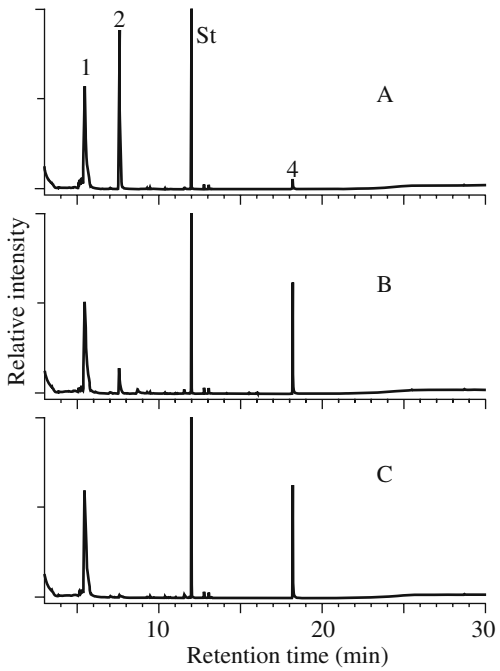


Fig. 4 Incubation of a mixture of benzoyl cyanide **2** and mandelonitrile **3**, with heat denatured "crude enzymes (macerated millipedes)". **a** 3 min after incubation; **b** 40 min after incubation; and **c** 85 min after incubation. **1**, benzaldehyde; **2**, benzoyl cyanide; **St**, n-tetradecane and **4**, mandelonitrile benzoate

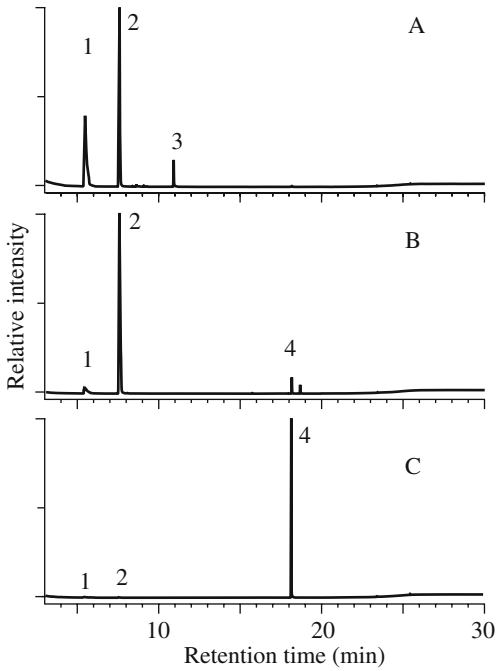


Fig. 5 A reaction between benzoyl cyanide **2** and mandelonitrile **3** under Schotten-Baumann conditions. **a** Control (mixture of benzoyl cyanide **2** and mandelonitrile **3**); **b** reaction after addition of a drop of water; **c** reaction after addition of a drop of pH 8 buffer (1*M* Tris-HCl buffer). **4**, mandelonitrile benzoate

(pHs of *Ne. tambanus tambanus* and *O. gracilis* were ca. 9.0); and 3) cyanide (**2**) is a potent reagent, equivalent to acid halide or acid anhydride, to produce esters or amides with alcohols or amines under basic conditions.

As a trial, one drop of pH 8.0 buffers was added to the neat mixture (less than 10 mg, transparent oil) of **2** and **3**. The mixture instantly turned milky-white, and produced **4** as a major product with no remaining trace of **2** or **3** (as **1**) (Fig. 5c). No reaction occurred with the addition of a drop of water to the same mixture, and a large amount of unreacted **2** was obtained together with a small amount of **4** (Fig. 5b, the nitrile (**3**, as **1**) appeared to be largely lost due to its solubility in water). In contrast, the mixture of **2** and **3** did not begin to react without alkaline conditions, even during both neat and heated conditions, whereas a dilute mixture in hexane seemed to react gradually during storage. As a whole, formation of **4** by shake-disturbance occurred as a result of the Schotten-Baumann reaction between **2** and **3** under basic conditions of bled bodily-fluids to release hydrogen cyanide instantly (Scheme 1).

The nitrile **3** is biosynthesized from phenylalanine (Duffey et al., 1974), and the directly sampled defensive secretion from a gland's orifice of *Ne. tambanus tambanus* by a glass capillary is composed only of **3** (Kuwahara et al., 2008). The cyanide (**2**, an oxidation product from **3** and extractable by hexane) is not involved in the biosynthetic sequence, thus suggesting transformation during or after discharge from the defensive gland. Similarly, the oxidized compound 2-methyl-3,4-methylenedioxyphenol (considered to be a reaction product between 2-methoxy-3-methyl-1, 4-benzoquinone and its hydroquinone), has been reported in a millipede *Acladocricus setigerus* (Diplopoda:Spirobolidida) (Wu et al., 2007). Although its production is explained chemically (Wu et al., 2007), it is reasonable to suggest the involvement of oxidation/reduction enzyme(s). In fact, previously we have demonstrated similar dehydrogenase (oxidase) activity in Astigmatid mite bodies suspended in hexane. It operates reversely to change citral (a mixture of neral and geranial) to its alcohols (Kuwahara et al., 1983), and has been characterized from *Carpoglyphus lactis* as a new class of dehydrogenase, with almost no homogeneity to currently known groups (Noge et al., 2008). Although the origin of **2** in millipedes remains unclear, the Schotten-Baumann reaction between **2** and **3** is concluded to be a new mechanism of instantaneous hydrogen cyanide release against enemy attack, emitting defensive secretions together with bled bodily fluids.

In Japan, a total of 14 polydesmoid millipedes have been studied based on their defensive secretions, among which eleven species are cyanogenic (listed in Table 1), with the remaining three, *Ni. nodulosa* Verhoeff (Ômura et al., 2002a), *Eu. elegans* Miyosi (Kuwahara et al., 2002), and *Eu. armatus* Miyosi (Kuwahara et al., 2003), identified as non-cyanogenic. The eleven cyanogenic species now are divided into two groups, with one group denoted by the first six species in Table 1, in which either the Schotten-Baumann reaction has been demonstrated to produce **4** together with hydrogen cyanide release, or its production is indicated by the presence of **2** and a detectable amount of **4** in the solvent extract. These millipedes belong either to Paradoxosomatidae, Xystodesmidae, or Polydesmidae.

The second group corresponds to the latter five species in Table 1. Although their defense secretions are composed of only **3**, a detectable amount of **4** is produced when a millipede is shake-disturbed with exogenously added benzoyl cyanide (**2**), as demonstrated in *O. gracilis* and *Cryptocorypha* sp. (Fig. 3 and Table 1). The millipedes from this second group belong either to Paradoxosomatidae, Xystodesmidae, Polydesmidae, or Pyrgodesmidae. Based solely on Japanese cyanogens millipedes, instantaneous hydrogen cyanide release by the Schotten-Baumann reaction, as a whole, might be distributed among roughly half the cyanogen millipedes that belong either to Paradoxosomatidae, Xystodesmidae, or Polydesmidae. Other than these species, defensive secretions **2** and **4** are known in millipedes belonging to three polydesmoid (Conner et al., 1977), as well as to twelve species that belong to Xystodesmidae (Duffey et al., 1977), whereas involvement of the present post-secretion Schotten-Baumann reaction has not been previously identified as a hydrogen cyanide release mechanism by millipedes. At present, no taxonomic evidence is available in the literature to support an evolutionary trend. However, the defensive mechanism might be a more advanced and evolved chemical defense than the natural or lyase-catalyzed dissociation of labile **3** to hydrogen cyanide and benzaldehyde (**1**), as in the case of *Harpaphe haydeniana* (Xystodesmidae: Polydesmida) (Duffey and Towers, 1978) (Scheme 1).

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Effects of Ingested Secondary Metabolites on the Immune Response of a Polyphagous Caterpillar *Grammia incorrupta*

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Abstract We considered the effects of plant secondary metabolites on the immune response, a key physiological defense of herbivores against pathogens and parasitoids. We tested the effect of host plant species and ingested iridoid glycosides on the immune response of the grazing, polyphagous caterpillar, *Grammia incorrupta* (Arctiidae). Individuals of *G. incorrupta* were fed either one of three plant diets with varying secondary metabolites, or an artificial diet with high or low concentrations of iridoid glycosides. An immune challenge was presented, followed by measurement of the encapsulation response. We failed to detect a significant difference in the immune response of *G. incorrupta* feeding on diets with varying concentrations of iridoid glycosides, or feeding on different host plants. However, the immune response was lower in caterpillars consuming the artificial diet compared to those consuming the plant diets. When caterpillar performance was measured, pupal weights were lower when caterpillars ingested high concentrations of iridoid glycosides due to a decrease in feeding efficiency. Overall, individuals of *G. incorrupta* that consumed different plant diets exhibited a high immune response with low variation. We conclude that the immune response of *G. incorrupta* is adapted to feeding on a variety of plants, which may contribute to the maintenance of this caterpillar's polyphagous habit.

Key Words Immune system · Encapsulation · Diet breadth · Iridoid glycosides · Sequestration · Secondary chemistry · Parasitism · Insect · Arctiidae

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Introduction

Insect herbivores rely on host plants to meet daily nutritional requirements needed for proper metabolism and growth. Beyond these basic physiological functions, host plant secondary metabolites may help structure herbivore interactions with natural enemies (Turlings et al., 1990; Dyer, 1995; Smilanich et al., 2009a). Detoxification or accumulation of these metabolites may incur physiological costs (Camara, 1997; Despres et al., 2007), leading to ecological and evolutionary consequences (Bowers, 1992; Nishida, 2002). A variety of studies have demonstrated that ingestion and sequestration of secondary metabolites renders herbivores unpalatable to predators such as birds, wasps, and ants (e.g., Bowers, 1992; Dyer, 1997; Vencl et al., 2005), thus providing protection from predators. However, ingestion of secondary metabolites also can increase susceptibility to natural enemies such as parasitoids because (1) chemically-defended herbivore hosts provide enemy-free space for parasitoid development (Gentry and Dyer, 2002), or (2) sequestration and/or detoxification of secondary metabolites alters herbivore immune responses (Smilanich et al., 2009a).

The insect immune response is an effective defense against parasitoids and pathogens (Beckage, 2008; Smilanich et al., 2009b). In this paper, we investigated

whether diet causes variation in the immune response, which could lead to vulnerability to parasitism. In particular, we presented an immune challenge to polyphagous, sequestering caterpillars that were consuming different levels of secondary metabolites.

The immune response consists of three main components: phagocytosis, nodule formation, and encapsulation (Carton et al., 2008). Encapsulation is the concerted action in which specialized immune cells (hemocytes) adhere to a large foreign body, such as a parasitoid or parasitoid egg, and build layers of cells that eventually are melanized. The cytotoxic effect of melanization and asphyxiation from encapsulation both contribute to killing the pathogen (Carton et al., 2008). Host plant chemistry can have negative, positive, or neutral effects on the immune response (Klemola et al., 2007, 2008; Bukovinszky et al., 2009; Smilanich et al., 2009a). Plant nutrients (e.g., protein) can enhance encapsulation and lysozyme-like antibacterial activity, but may have little effect on other immune system components like phenoloxidase activity (Lee et al., 2008; Povey et al., 2009). Ingestion and sequestration of some plant secondary metabolites (e.g., iridoid glycosides) can weaken the immune response by directly interfering with the melanization component of immunity (Smilanich et al., 2009a), whereas other metabolites (e.g., hydrolyzable tannins) may act indirectly on immunity via reductions in herbivore performance (Haviola et al., 2007; Yang et al., 2008). In contrast, carotenoids, flavonoids, and synthetic chemicals may enhance immunity, perhaps by quenching harmful oxygen species (Babin et al., 2010).

Given the possibility that plant chemistry may have a negative effect on the immune response, herbivores face the dual challenge of digesting and assimilating plants of variable quality and at the same time maintaining the immune response in order to evade parasitoids. Here, we used experiments with the grazing generalist caterpillar, *Grammia incorrupta* Hy. Edwards (Arctiidae), to address the following questions: (1) what are the effects of three host plant species with different nutrient and secondary chemical profiles on the immune response, and (2) what are the effects of iridoid glycosides (IGs) added at different levels to a standard diet on the immune response? *Grammia incorrupta* larvae sequester pyrrolizidine alkaloids (0.3 to 2.7 mg/g dry wgt, Hartmann et al., 2005) and low levels of IGs (Bowers, 2009).

To address these questions, larvae were reared on three different host plants: *Plantago major* (Plantaginaceae), which has low concentrations of one IG, aucubin (Barton and Bowers, 2006); *P. lanceolata*, which has high concentrations of two IGs, aucubin and catalpol (Bowers and Stamp, 1992), and *Taraxacum officinale* (Asteraceae), which has no IGs, but does contain flavonoids, phenolics, and sesquiterpenes (Schutz et al., 2006). Larvae were presented with an immune challenge and the immune response was analyzed. To more specifically examine the effects of IGs, larvae were fed artificial diets that contained extracted IGs (aucubin and catalpol) and were then presented with an immune challenge. For the artificial diet experiment, we also measured feeding efficiency in order to test for a relationship between food utilization and the immune response. Based upon previous work (Smilanich et al., 2009a), we predicted that ingestion of IGs would negatively affect the immune response, and that host plants with the highest content of IGs would be detrimental to the immune response.

Methods and Materials

Experimental Overview Experiments with *G. incorrupta* were performed at Tulane University, New Orleans, LA, USA during fall 2006, and at University of Colorado, Boulder, CO, USA during fall 2008. Egg masses were obtained from an established lab colony at Wesleyan University, Middletown, CT, USA (courtesy of M.S. Singer). The first experiment tested the effects of host plant species on the immune response. The second experiment tested the effects of high and low concentrations of IGs on the immune response and feeding efficiency by using an artificial diet with added IG extract.

Immune Assay To measure the immune response, *G. incorrupta* caterpillars were injected with Sephadex beads as a proxy for parasitization (Lavine and Beckage, 1996; Rantala and Roff, 2007; Smilanich et al., 2009a,b). The beads (Sigma-Aldrich, Sephadex A25, 40–120 μm) were dyed red using 0.1% Congo red and were suspended in Ringer's solution so that 5–10 beads could be injected into the base of the third proleg (Lavine and Beckage, 1996). Caterpillars then were returned to their test diets and after 24 hr were freeze-killed. Caterpillars were dissected in 95% methanol, and beads were photographed with a camera mounted on a dissection microscope focused at 80× magnification (Carl Zeiss Discovery V.8, AxioVision software). Since the beads were dyed red before injecting them into the caterpillars, we quantitated melanization by measuring the red value (r-value, Adobe Photoshop ver. 6.0), a scale ranging from 0–255, where 0 = pure gray, and 255 = pure red, for each bead. The mean r-value for all the beads from each caterpillar was statistically compared between treatments, using ANOVA with r-value as the dependent variable and treatment as the independent variable. The r-value was transformed into percent melanization [1−(r-value/maximum r-value)] where the maximum r-value is 255 for a non-injected, unmelanized bead.

Experiments with Three Host Plants Caterpillars were randomly assigned to one of three diets: *T. officinale*, *P. lanceolata,* or *P. major*. *Plantago major* contains only one iridoid glycoside, aucubin, at relatively low levels (0.2–1.0% dry weight) (Barton and Bowers, 2006), while *P. lanceolata* contains both aucubin and its derivative catalpol at much higher concentrations, ranging from 5–12% dry weight (Bowers and Stamp, 1992). *Taraxacum officinale* does not contain iridoid glycosides, but does contain flavonoids generally considered less toxic to herbivores (Harborne, 1991; Schutz et al., 2006). Newly hatched larvae were reared in groups of five in plastic rearing cups in growth chambers with 16:8 h L:D control, and 25°C daytime, 20°C nighttime temperatures. Plants were collected from Boulder County, CO, USA, soaked in dilute bleach solution for approximately 10 min, rinsed three times, and spun dry in a salad spinner before they were offered to caterpillars *ad libitum*. After molting to the 6th instar, 25 caterpillars feeding on each of the three host plants were removed in order to test the immune response. An additional 20 *G. incorrupta* caterpillars from each of the *Plantago* treatments were removed, injected, and immediately frozen for assessment of IG sequestration. Ten individuals from each host plant were dissected in methanol, and 10 were left intact in order to determine whether the dissection procedure affected IG sequestration. Five subsets of leaves of *P. major* and *P. lanceolata* from the material fed to caterpillars were dried at 50°C to a constant weight, ground to a fine powder, and subsampled to quantify IGs.

Chemical Analysis Iridoid glycosides in plant material and caterpillars reared on *P. major* and *P. lanceolata* were quantified by gas chromatography. Frozen caterpillars were ground in 5 ml of 95% methanol and extracted overnight. Those caterpillars that had been previously dissected were extracted overnight in the methanol solution utilized for the dissection. A subsample (25 mg) of dried plant material was extracted in 5 ml of 95% methanol overnight. Caterpillar and leaf samples were filtered, and the methanol was removed by evaporation. An internal standard (phenyl-β-D-glucose, Sigma-Aldrich, St. Louis, MO, USA) was added, and the sample was partitioned between water and ether in order to remove hydrophobic compounds. The water phase was collected, evaporated to dryness, and the residue was dissolved in 1 ml methanol. An aliquot of 100 μl was dried overnight, derivitized with 100 μl Tri-Sil-Z (Pierce Chemical Company, Rockford, IL, USA) and injected (1 μl) into a Hewlett Packard 5890A gas chromatograph equipped with a FID detector for separation as described by Bowers and Stamp (1992). The two iridoid glycosides, aucubin and catalpol, were identified by comparison with standards of the pure compounds that were extracted and isolated from plant material (Camara, 1996) and quantified using Agilent Chem Station software.

Artificial Diet Experiment This experiment was designed to measure the effects of IGs on the immune response while holding all other diet variables equal. Thus, we used an artificial diet (Lei and Camara, 1999) to which we added known concentrations of IGs. Iridoid glycosides were extracted and purified from *P. lanceolata* collected from Southern Alabama (Camara, 1996). The crude extract comprised 8% IG making it impossible to adjust the artificial diet to 12% IG, which is the upper limit found in natural plant populations. Crude extract was dissolved in water and added to the artificial diet mix to yield a 1% (low concentration) and a 5% (high concentration) IG diet. Newly hatched caterpillars were assigned randomly to high or low diets and placed in individual plastic rearing cups (30 per treatment) with food. Rearing cups were kept in a walk-in growth chamber with environmental conditions set at 12:12 h L:D at 23°C. The immune response was tested at the beginning of 6th instar.

Feeding Efficiency Experiments For the artificial diet experiment, feeding efficiency was calculated using the standard gravimetric method (Waldbauer, 1968). All measurements were obtained over a standardized time interval starting at third instar and ending when caterpillars molted into 6th instar. Measurements included food mass, body mass, and fecal mass each day. In addition, samples of larvae and diet were dried and weighed at the beginning and the end of the experiment. Growth rate and feeding efficiency measures were analyzed using MANOVA where measurements at each instar were considered separate response variables (i.e., multivariate repeated measures). The following indices were calculated for each caterpillar:

Efficiency of conversion of ingested food (**ECI**) = larval dry weight gain/dry weight of food consumed
Approximate digestibility (**AD**) = (dry weight of food consumed − dry weight of frass)/dry weight of food consumed
Efficiency of conversion of digested food (**ECD**) = larval dry weight gain/(dry weight of food consumed − dry weight of frass)
Growth Rate (**GR**) = larval dry weight gain/(duration of feeding period*mean weight during growth interval).

(1)

Results

Three Host Plants Individuals of *G. incorrupta* that fed on the three different host plants did not significantly differ in their immune response ($F_{2,4}$=2.69, N=42, P=0.081) (Fig. 1a). Within each species, the iridoid glycoside content was quite variable but overall *P. lanceolata* had a higher IG content than *P. major* (*t*-test, P=0.015) (Fig. 2). *Grammia incorrupta* larvae sequestered very low levels of IGs (Fig. 2) and fewer than 40% of the larvae tested contained detectable amounts. To determine whether dissection of larvae affected IG content by promoting breakdown of the IGs, we performed a two-way ANOVA on the arcsine square root transformed proportion of total IGs in larvae, with host plant and dissection as the main effects. For this analysis, we used only those larvae in which IGs were detected (only aucubin for larvae reared on *P. major* and both aucubin and catalpol for larvae reared on *P. lanceolata*). The amount sequestered was not affected by host plant species ($F_{1,10}$=2.52, P=0.143) or by dissection ($F_{1,10}$=3.84, P=0.079), although there was a trend for dissected larvae to contain higher levels of IGs (Fig. 2), nor was there an interaction ($F_{1,10}$=0.220, P=0.649).

Artificial Diet When individuals of *G. incorrupta* were fed IGs in an artificial diet at 5% and 1% concentrations, there was no significant difference in the melanization response ($F_{1,70}$=0.01, N=71, P=0.907) (Fig. 1b). Pupal weights were higher on the 1% iridoid diet compared to the 5% iridoid diet ($F_{1,37}$=10.76, N=38, P=0.002). There were no significant differences in growth rates across all instars (Table 1), indicating that the higher pupal weights on the 1% diet were not a result of slower growth, which could allow more time to feed. The difference in pupal weights may be due to significant differences in the digestion indices. The ECI and ECD differed significantly between diets (Table 1). For both indices, the mean consumption (dry weight of food consumed / average larval dry weight during interval) for caterpillars feeding on the 1% IG diet were higher. However, AD did not significantly differ between caterpillars feeding on the 1% and the 5% diets (Table 1). These results indicate that while caterpillars may be assimilating nutrients from food (AD) efficiently on both diets, they are less efficient at converting the assimilated nutrients into caterpillar biomass on the 5% iridoid diet, thus resulting in lower pupal weights.

Discussion

Our initial hypothesis that ingestion and sequestration of iridoid glycosides would cause significant decreases in the immune response was not supported. Sequestration was a poor predictor of variation in the immune response, since overall sequestration was low, and many individuals had undetectable levels of IGs. Poor sequestration ability also was found in another generalist arctiid, *Estigmene acrea* (Lampert and Bowers, 2010). Individuals feeding on diets with high and low concentrations of IGs exhibited almost identical immune responses. The overall high levels of melanization on the plant diets, and the low amount of variation among all diets suggest that individuals of *G. incorrupta* have a reliably strong immune response.

There was, however, a 37% difference in melanization score between caterpillars feeding on the artificial diet and caterpillars feeding on the plant diets. The relatively lower degree of melanization in larvae fed on the artificial diets may be due to the lack of essential substrates necessary for the phenoloxidase cascade to occur. The phenoloxidase cascade promotes the melanization process that darkens the cells encapsulating a foreign object (Beckage, 2008). The artificial diet contains only essential nutrients so that caterpillars are not ingesting pigments or other plant components that may be required for melanization.

Immune responses can be highly variable in insects (Rolff and Siva-Jothy, 2003). This variation is attributed to environmental stress (van Ooik et al., 2008), diet (Smilanich et al., 2009a), genetic variation (Lazzaro et al., 2004), or predation pressure (Slos et al., 2009). In our study, the only variation in the melanization response was associated with artificial diets vs. leaf diets. The results with

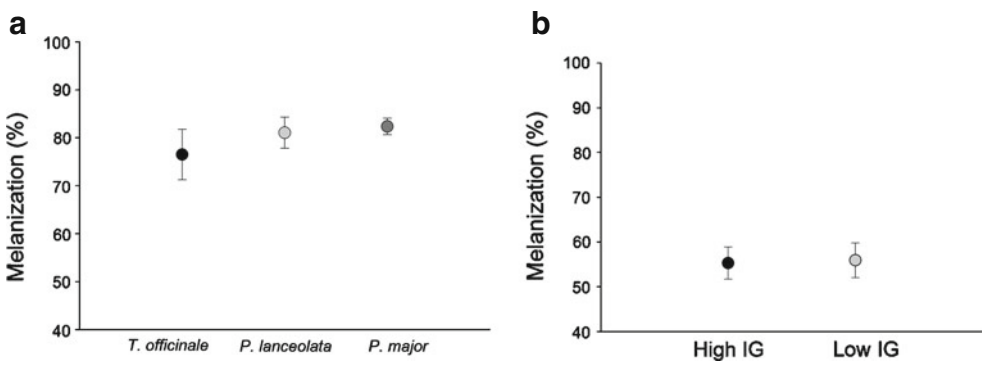


Fig. 1 Response to immune challenge for *Grammia incorrupta* consuming plant diets (*Taraxacum officinale, Plantago lanceolata*, or *Plantago major*) (**a**) or iridoid glycoside (IG)-containing artificial diets (**b**). The immune response was measured as % melanization of injected particles

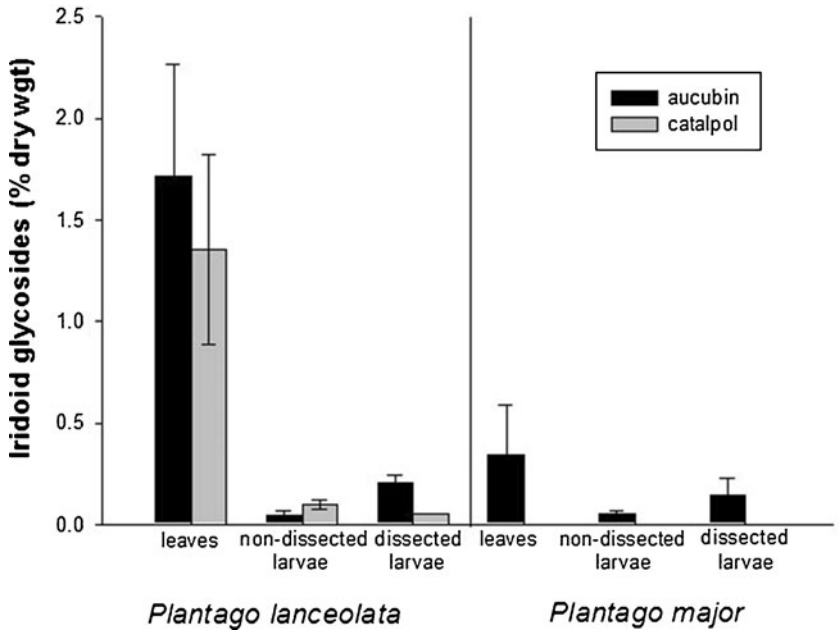


Fig. 2 Level of iridoid glycosides in *Plantago lanceolata* and *P. major*, and in intact or dissected *Grammia incorrupta* fed on either *P. lanceolata* or *P. major*

leaf diets suggest that high concentrations of IGs in the diet are not directly toxic to the immune system in this species. One reason for the low variation among diets in this study may be because this species is not efficient at sequestering IGs. In a previous study, we found that the specialist buckeye caterpillar, *Junonia coenia* (Nymphalidae), which can sequester IGs in concentrations as high as 25% dry weight (Bowers and Stamp, 1997), had a significantly lower melanization response when sequestering high concentrations of IGs (Smilanich et al., 2009a). Since *G. incorrupta* inefficiently sequesters IGs, the impact of the compounds on the immune response is probably minimal, thus leading to the small effect observed in our study.

Consumption of the 5% IG diet caused a significant decrease in pupal weights compared to the 1% IG diet. This effect most likely was driven by differences in the consumption indices, ECI and ECD, which were dramatically lower (36%) on the high IG diet. In our study of immune-challenged insects, feeding efficiency can give an approximation of the metabolic costs of feeding on diets with high concentrations of secondary metabolites coupled with mounting an immune response. The ECI measures the overall efficiency with which the caterpillar assimilates food into biomass. This measurement is broken down into the AD and ECD, where AD measures the proportion of nutrients that are assimilated from the ingested food (predigestion), and ECD measures the proportion of assimilated food that is turned into caterpillar biomass. If individuals on the high IG diet are consuming less and converting less of the digested food to biomass, then a decrease in pupal weights is expected. This difference in pupal weights was not correlated with growth rate or development time since there was no significant difference between diets for these variables. Instead, the increase of IG concentration in the caterpillar's diet negatively affected pupal weights by interference with consumption and post-digestive ability. Since pupal weights are correlated with adult fecundity (Gilbert et al., 1984), *G. incorrupta* fecundity may be lower when

Table 1 Feeding efficiency parameters from the artificial diet experiment

	F	*P*	1% Iridoid glycoside diet			5% Iridoid glycoside diet		
			Mean	SEM	*N*	Mean	SEM	*N*
GR	0.48	0.49	0.009	0.001	42	0.006	0.0007	47
ECI	8.34	0.006	3.10	0.49	42	1.96	0.19	47
AD	0.05	0.82	92.40	0.45	42	92.07	0.41	47
ECD	8.29	0.006	3.34	0.53	42	2.12	0.20	47

MANOVA was used to analyze GR, growth rate; ECI, efficiency of conversion of ingested food; AD, approximate digestibility; ECD efficiency of conversion of digested food. Mean values are reported (standard error of the mean, *SEM;* sample size, *n*). *F* is reported as Wilks' Lambda

feeding on plants with high concentrations of secondary metabolites. However, if individuals of *G. incorrupta* are able to switch hosts easily, then continued feeding on a nutritionally poor plant is unlikely unless individuals are parasitized (Singer et al., 2009).

The ability to switch host plants and feed on plants with suboptimal quality can be an asset for an insect challenged by a parasitoid (Karban and English-Loeb, 1997; Singer et al., 2009). Switching hosts as a defense against parasitoids may outweigh any developmental costs suffered by feeding on an inferior host. Our study provides further support that being a polyphagous feeder is advantageous by demonstrating that the immune response did not vary among three plant species, which are likely to have different nutritional qualities as well as differences in secondary metabolite contents.

The evolution of herbivore diet breadth is complex and influenced by many variables (Singer and Stireman, 2005). Here, we found that the immune response, which is one of the most effective defenses against parasitoids, did not vary among a select set of host plant species and was not affected by one group of secondary metabolites, iridioid glycosides. This lack of variability may allow individuals to feed on a variety of plants without detrimental effects on the immune response. Recent work has demonstrated that pyrrolizidine alkaloids, which are actively sequestered by *G. incorrupta*, also do not affect the immune response (Smilanich et al., 2011). In light of these results, we suggest that this polyphagous caterpillar is well adapted to feeding on host plants of variable quality with no detrimental effect on its immune response.

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Epicuticle Lipids Mediate Mate Recognition in *Triatoma infestans*

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Abstract Epicuticular lipids are contact cues in intraspecific chemical communication in insects, both for aggregation and sexual behavior. Triatomine bugs are vectors of the parasite *Trypanosoma cruzi*, the cause of Chagas disease. In *Triatoma infestans*, the major epicuticular lipids are hydrocarbons, fatty alcohols, and free and esterified fatty acids. Previously, we found that epicuticular lipid extracts, or selected fatty acid components, trigger aggregation and arrestment behavior in this bug. Using headspace solid phase microextraction, we found no sexual dimorphism in epicuticular hydrocarbons, but found female-specific fatty alcohols (eicosanol and docosanol). The role of epicuticular lipids in *T. infestans* copulation behavior was tested by observing male responses to live or various treatments of freeze-killed females. We report that hexane-soluble contact cues on females trigger copulation by males. Freeze-killed intact females were attractive to males, but no response was observed when males were exposed to hexane-washed females. Responses were partially recovered when epicuticular extract was applied to the dorsal surface of dead, hexane-washed females. One female equivalent of docosanol, evoked similar responses.

Key Words Contact sex pheromone · Mating behavior · Fatty alcohols · Hydrocarbons · Chagas disease vectors · Hemiptera · Reduviidae

Introduction

Triatoma infestans (Hemiptera: Reduviidae) is a major vector of Chagas disease in South America, especially in the 1.3 million km^2 Gran Chaco geographic region, which has a mostly rural human population (Schofield and Kabayo, 2008). Late nymphs and adults are the major targets in vector control strategies, as they are more likely to transmit the disease. Attractants for this bug could help in the development of new control methods by using these chemicals as components of traps that monitor or "attract and kill". Recently, a "trap and kill" device that used entomopathogenic fungi, was shown to be successful against *T. infestans* (Pedrini et al., 2009).

Epicuticular lipids are known to mediate mate recognition in many insect species (Schal et al., 1990; Ferveur et al., 1996; Ginzel et al., 2006; Barbour et al., 2007; Rutledge et al., 2009). *Triatoma infestans* and other triatomines exhibit characteristic aggregation (Juárez et al., 2008; Lorenzo Figueiras et al., 2009), although no evidence for a contact sex or aggregation pheromone has been described. The epicuticular lipids of *T. infestans* are a complex mixture of long-chain hydrocarbons and oxygenated components, mostly fatty alcohols, waxes, and free and esterified fatty acids, which protect insects against chemical and biological attack (Juárez, 1994, 1995; Juárez and Calderón-Fernández, 2007; Pedrini et al., 2009). Epicuticular hydrocarbons are

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sexual dimorphic in *Triatoma sordida*, although no sex difference was found in *T. infestans* (Juárez and Calderón-Fernández, 2007).

Here, we tested the hypothesis that contact pheromones mediate mate recognition in *T. infestans*. We examined the behavioral role of epicuticular lipids and show, for the first time, that these lipids elicit male mating attempts. We also report the identification of a female-specific fatty alcohol component that is capable of eliciting this behavior.

Methods and Materials

Insects Triatoma infestans were from a colony at the INIBIOLP, reared at 28°C, 50–60% RH. Adults from a small field colony were collected and used in one experiment. Virgin adults were collected daily and maintained separately. Insects were fed on domestic chickens 2–3 d after last molt, and used 10–12 d thereafter. Chickens were housed and managed under standard laboratory conditions approved by INIBIOLP's Animal Care and Use Committee.

Solvent Extraction and Head Space Solid Phase Microextraction (HS-SPME) Sampling Epicuticular lipids were extracted from single-sex groups of three bugs in redistilled hexane (6 ml g^{-1}, 3 times 5 min. each). The extract was concentrated under a stream of nitrogen. Hydrocarbons were fractionated by silica gel column chromatography, as described (Calderón-Fernández et al., 2011), using hexane as eluent.

For HS-SPME, hexane extracts of epicuticular lipids were deposited inside 10 ml crimp top vials (Supelco, Bellefonte, PA, USA), and the solvent was evaporated by a nitrogen stream. Vials were sealed and the residual lipids sampled at 150°C by HS-SPME for 20 min., using a 65 μm polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB) (Supelco) inserted through the septum.

Chemical Analysis Both hydrocarbon and epicuticular lipid samples were analyzed by coupled gas chromatography–mass spectrometry (GC-MS) using electron impact ionization (70 eV). A Hewlett-Packard 6890 GC, equipped with a DB-5MS capillary column (30 m×0.25 mm×0.25 μm film; J&W Scientific, Folsom, CA, USA), interfaced to an Agilent 5975 mass selective detector, was used for the analyses. Samples were injected either splitlessly or directly (SPME), with helium as carrier gas. The column oven was programmed from 50–180°C (at 20°C.min^{-1}), then to 310°C (at 3°C.min^{-1}). Transfer line and quadrupole were held at 320°C and 150°C, respectively. Peak areas were calculated and expressed as a percentage of total peak area. Interpretation of hydrocarbon mass spectra was performed as described in Juárez et al. (2001). Spectra of other lipids were compared to data from MS libraries (NIST/EPA/NIH, NIST 05 and ADAMS 07). Fatty alcohols were identified by interpretation of their mass spectral fragmentation and by a close match of their mass spectra and retention times with those of authentic standards. Absolute amounts of fatty alcohols were quantified by flame ionization detection and calculated relative to eicosanol as an external standard; response was linear in the concentration range tested.

Bioassays Bioassays were performed in the late evening in plastic Petri dishes (13 cm diam.×12 cm height), generally using paired virgin males and females (alive or dead). Live males and females (N=68 each sex) were used to characterize the mating behavior of adults. A further experiment, using live females, tested the role of antennal contact in mating by covering the antennae of males with eyelash gel.

To test whether a contact pheromone was involved in mate recognition, females were freeze-killed (−20°C, 30 min) and allowed to warm to room temperature. These dead females were tested to determine whether recognition cues remained unchanged after killing, and therefore independent of female behavior. Next, epicuticular lipids were extracted from a dead female, as above, and the female was air-dried for ~30 min. The responses of individual males, were tested to the solvent-washed female. Finally, we loaded 1 or 3 female equivalents (FE) of lipid extract (in 20 μl hexane) onto a dead female. Each dead female was presented to 1–5 males (N=84 males and 48 females). The response of a male was observed by eye or recorded with a video camera over 30 min. A trial was scored as a "positive response" if the male, after antennal contact, stopped walking (arrest behavior), aligned with the female, and mounted and grasped her (copulation attempt). If the male showed no response after antennal contact, it was scored as "no response". Male responses were tested to live females, dead and intact females, dead and hexane-washed females, and dead, washed and lipid-reconstituted females.

To test the role of female-specific components in mate recognition, hexane solutions of 1 FE of docosanol and eicosanol (Sigma Aldrich, St. Louis, MO, USA) either individually, or combined, were placed on 2 cm long, polypropylene half cylinders, cut from 1 ml micropipette tips. Males were exposed to treated tips or control tips (hexane), in a arena similar to that described. A positive response was scored when a male mounted the tip.

The responses to treatments were tested by Fisher's exact test (Sokal and Rohlf, 2001). Differences were considered to be significant if the probability of their occurrence by chance was less than 5%.

Results

Hexane-extracts of epicuticular lipids of mature virgin *T. infestans* sampled by HS-SPME had a high abundance of saturated hydrocarbon components, mostly straight chain from 21 to 33 carbons, together with straight chain fatty alcohols of 20–30 carbons. Other minor components were fatty acids and fatty aldehydes (Fig. 1 and Table 1). No evident sexual dimorphism was observed for these components, except female extracts contained more than 7% of eicosanol and docosanol, whereas male extract had only trace amounts of these compounds. One equivalent of docosanol and eicosanol in a female corresponded to 14.1±3.9 ng and 6.1±0.8 ng, respectively. Females also had higher proportions of tetracosanol, which coeluted with *n*- heptacosane (Fig. 1 and Table 1).

Male *T. infestans* only attempted to mate (57.6% of total males tested attempted mating, N=66) with live females after contacting them with their antennae. After initial wandering in the container, males stopped, turned their antennae toward the female and contacted her with them, usually on the abdominal dorsal surface and the thorax (step 1). As described (Manrique and Lazzari, 1994), males aligned their body to the female, mounted and grasped her (step 2). After a male assumed a dorsolateral position and exposed his genitalia, copulation commenced (step 3). Most

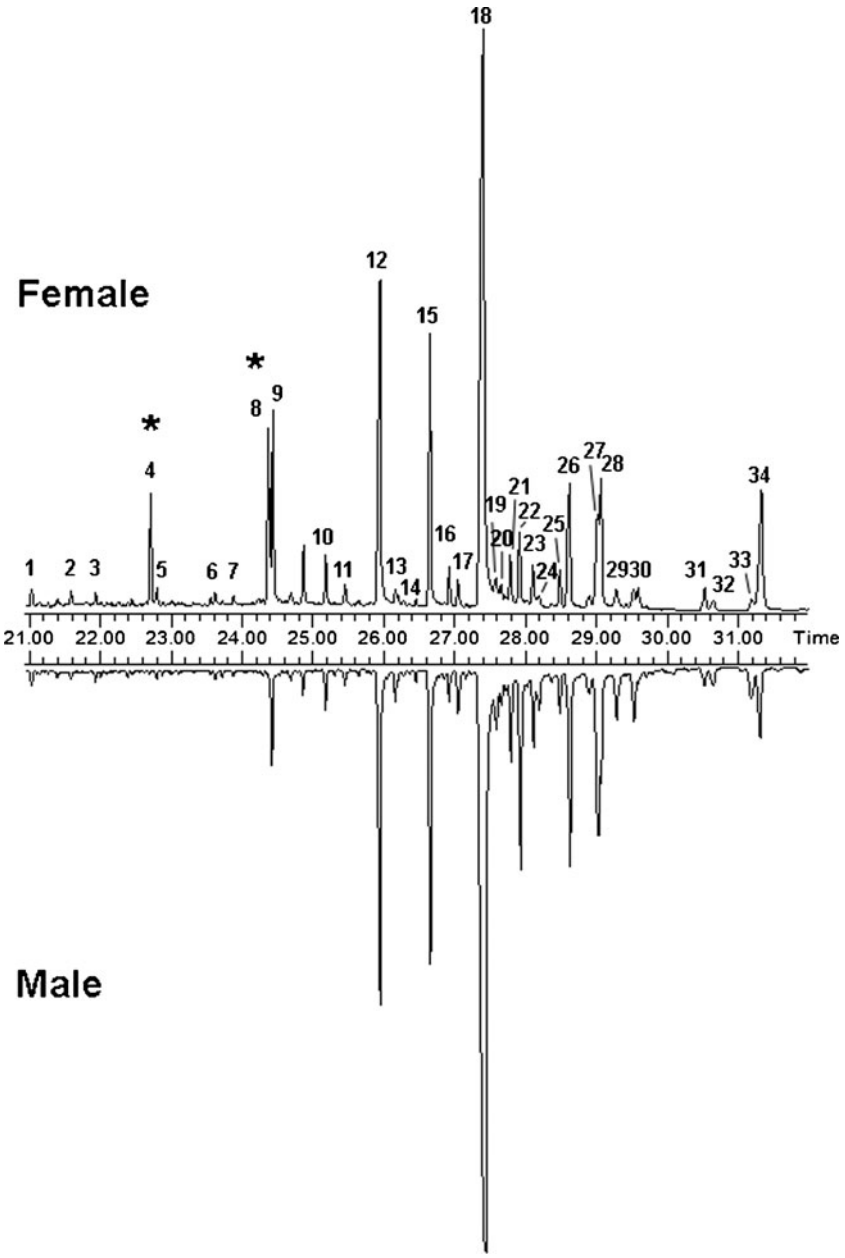


Fig. 1 Total ion mass chromatograms obtained by solid phase microextraction of epicuticular lipids of virgin female (*top*) and male (*bottom*) *Triatoma infestans*. Eicosanol and docosanol (marked with asterisk) were found in large abundance only in females. The identities and relative proportions of major components are given in Table 1

Table 1 Major epicuticular lipid compounds found in solid phase microextraction sampling of hexane. Extracts of virgin adult female and male *Triatoma infestans*

Peak #	KI	Compound	Females		Males		Diagnostic *m/z*
			Mean[a]	SD	Mean[a]	SD	
1	2100	*n*-Heneicosane	0.37	0.12	0.37	0.13	43, 57, 71, 85, 296
2	2162	Octadecanoic acid	0.41	0.26	0.32	0.12	43, 57, 73, 129, 185, 241, 284
3	2200	*n*-Docosane	0.26	0.08	0.26	0.09	43, 57, 71, 85, 310
4	2290	Eicosanol	3.14	0.36	0.04	0.06	43, 55, 69, 83, 97, 111, 252, 280
5	2300	*n*-Tricosane	0.31	0.05	0.28	0.10	43, 57, 71, 85, 324
6	2400	*n*-Tetracosane	0.37	0.09	0.33	0.09	43, 57, 71, 85, 338
7	2432	Docosanal	0.17	0.14	0.11	0.08	43, 57, 71, 82, 96, 111, 306, 324
8	2494	Docosanol	5.12	0.94	0.05	0.04	43, 55, 69, 83, 97, 111, 280, 308
9	2500	*n*-Pentacosane	2.40	1.79	2.15	1.14	43, 57, 71, 85, 352
10	2600	*n*-Hexacosane	0.90	0.39	0.83	0.05	43, 57, 71, 85, 366
11	2636	Tetracosanal	0.69	0.09	0.60	0.35	43, 57, 71, 82, 96, 111, 334, 352
12	2700	Tetracosanol[b] + *n*-Heptacosane	11.12	1.62	10.14	0.88	43, 55, 69, 83, 97, 111, 308, 336; 43, 57, 71, 85, 380
13	2730	11-; 13-methylheptacosane	0.48	0.11	0.75	0.16	196/197, 224/225, 168/169, 252/253
14	2772	3-Methylheptacosane	0.20	0.04	0.24	0.02	56/57, 336/337, 364/365
15	2800	*n*-Octacosane	7.16	0.74	8.38	1.62	43, 57, 71, 85, 394
16	2836	Hexacosanal	1.40	0.31	1.05	0.52	43, 57, 71, 82, 96, 111, 362, 380
17	2852	4-methyloctacosane	0.76	0.07	0.73	0.49	70/71, 365, 393
18	2900	*n*-Nonacosane	33.81	1.88	34.34	5.58	43, 57, 71, 85, 408
19	2925	13-, 11-, 15-Methylnonacosane	0.56	0.11	0.89	0.07	196/197, 252/253, 168/169, 280/281, 224/225
20	2936	7-Methylnonacosane	0.35	0.09	0.54	0.20	112/113, 336/337
21	2954	11,15-Dimethylnonacosane	1.27	0.08	1.80	0.61	168/169, 224/225, 239, 295
22	2972	3-Methylnonacosane	3.36	1.12	5.59	0.93	56/57, 393, 407
23	3000	*n*-Triacontane	1.08	0.07	1.60	0.23	43, 57, 71, 85, 422
24	3009	3,7-Dimethylnonacosane	0.70	0.48	1.26	0.68	127, 336/337, 407, 308/309, 379
25	3041	Octacosanal	1.78	0.58	1.40	0.44	43, 57, 71, 82, 96, 111, 390, 408
26	3056	4-Methyltriacontane	4.39	0.53	4.69	1.29	70/71, 393, 421
27	3100	*n*-Hentriacontane	3.83	0.24	5.97	1.35	43, 57, 71, 85, 436
28	3104	Octacosanol	4.04	0.41	3.74	2.13	43, 55, 69, 83, 97, 111, 364, 392
29	3124	11-, 13-, 15-Methylhentriacontane	0.86	0.12	1.26	0.24	168/169, 308/309, 196/197, 280/281, 224/225, 252/253
30	3147	11,15-; 13,17-; 11, 21-Dimethylhentriacontane	0.99	0.15	1.63	0.07	168/169, 239, 252/253, 323, 196/197, 224/225, 267, 295
31	3238	Triacontanal	1.45	0.37	1.12	0.31	43, 57, 71, 82, 96, 111, 418, 436
32	3250	4-Methyldotriacontane	0.49	0.10	0.55	0.25	70/71, 421, 449
33	3300	*n*-Tritriacontane	0.82	0.20	1.06	0.56	43, 57, 71, 85, 464
34	3313	Triacontanol	5.00	1.32	5.94	4.34	43, 55, 69, 83, 97, 111, 392, 420

Peak numbers correspond to peaks in Fig. 1

Males and females were analyzed separately

[a] Means of four groups of three individuals each

[b] Tetracosanol amount is roughly 50% and 26% of the overlapping peaks in females and males, respectively

(98.5%) males that reached step 2 also completed copulation. The time to first antennal contact, varied from 0.16 to 5 min (mean = 1.25 ± 1.31 min, N=13), and to mating attempt, from 0.33 to 12 min (4.27±4.63 min, N=10). The percentage of mating attempts by previously mated males (62.5%, N=8, unmated for 2 d) was not different from that of virgin males. No males (N=10) that had their antennae covered with an eyelash gel attempted mating

Freeze-killed females elicited 61.0% of males to attempt mating (N=41) (Fig. 2), not different (P=0.836) to the percentage elicited by live females, suggesting that active female behavioral cues were not involved. Hexane-washed female bodies (N=42) elicited no response from males, different from that elicited by intact, freeze-killed females (P<0.001). When epicuticular lipids (1FE) were loaded onto dead, washed females, 41.2% of males responded, differently (P=0.001, N=17) from that to dead, washed females only. At a higher dose (3 FE) of extract, 53.8% (N=26) of males responded, not different (P=0.536) from the percentage that responded to the lower dose (Fig. 2). Responses to dead and intact or reconstituted freeze-killed females were not different at either of the 2 doses tested (3 FE, P=0.246; 1 FE, P=0.616).

Triatoma infestans from a small field colony were also tested. Freeze-killed females elicited mating attempts from 100% of males tested (N=10), solvent-washed females evoked no males (N=9) to respond, and epicuticular lipid-reconstituted females (1 FE) elicited mating attempts from 57.1% of males (N=14). One FE of docosanol elicited 46.7% of males (N=30) to attempt copulation with the treated pipette tip. This percentage was different (P=0.025) from that observed by using 1 FE of eicosanol (16.7% of males, N=30) (Fig. 3). The percentage responses of males to docosanol (1 FE) were similar to those to docosanol plus eicosanol (1 FE each), either on a polypropylene tip (50%, P=0.811, N=38) (Fig. 3), or on hexane-washed females (61.1%, P=0.334, N=18). Control tips elicited no response from males, differing from responses to eicosanol (P=0.008), docosanol (P<0.001), and eicosanol plus docosanol (P<0.001).



Fig. 2 Responses of *Triatoma infestans* males to females with different treatments: freeze-killed, extracted with solvent, or addition of 1 (*white*) or 3 female equivalents (FE; *gray*) of epicuticular lipid extract following extraction with solvent (reconsitution). Forty one males and 27 females were used both in the freeze-killed and solvent-extracted treatments, and 43 males and 21 females in the reconstitution treatment. Different letters indicate differences (P≤0.05) among treatments by Fisher's exact test. The letter atop the reconstitution bar applies to both 1 and 3 FE

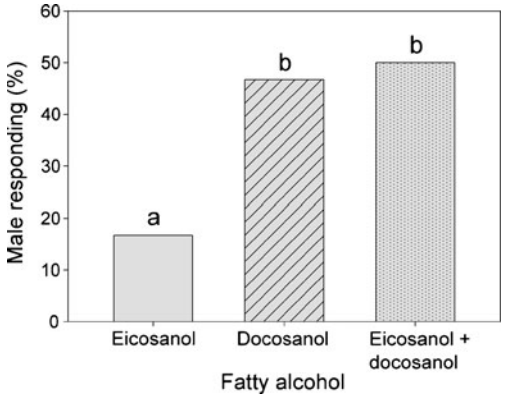


Fig. 3 Responses of *Triatoma infestans* males to polypropylene pipette tips treated with 1 female equivalent of eicosanol (*plain*), docosanol (*lines*), or 1 equivalent each of eicosanol plus docosanol (*dots*). Thirty males were tested to each individual alcohol treatment and 38 to the mixture. Different letters indicate differences (P≤0.05) among treatments by Fisher's exact test

Discussion

Previous analyses of cuticular hydrocarbons of *T. infestans* found saturated, mostly odd-numbered, straight chain compounds of 23–33 carbons, together with methyl-branched compounds of 29 or more carbons (Juárez and Blomquist, 1993). No evident sexual dimorphism in epicuticular hydrocarbons was detected in bugs of unknown mating status (Juárez and Brenner, 1985). In the present study, our analyses of extracts of virgin males and females of the same age also found no sexual dimorphism in the hydrocarbon fraction. However, our HS-SPME sampling allowed us to detect various oxygenated compounds in the epicuticular lipid extracts of *T. infestans*. A whole series of even-numbered fatty aldehydes of 22 to 30 carbons were novel, and present in both sexes. Of particular note were two female-specific fatty alcohols, docosanol and eicosanol. Previous studies, using solvent extracts, showed that triacontanol and dotriacontanol were the major fatty alcohol components in both females and males (Juárez and Calderón-Fernández, 2007).

Free fatty alcohols are not commonly found in epicuticular lipids of insects, although high molecular weight alcohols have been reported in honeybees (Blomquist et al., 1980) and triatomines (Juárez and Calderón-Fernández, 2007). High molecular weight alcohols also have been reported in the defensive secretions of scale insects (Buckner, 1993; Byrne and Hardley, 1988). Typically, insects more commonly produce lower molecular weight alcohols. Honeybees produce alcohols of 17–22 carbons, which induce arrestment in parasitic varroa mites (Donze et al., 1998). A mixture of short chain alcohols, their acetate derivatives, and (*Z*)-11-eicosenol are components of bee alarm pheromones (Pickett et al., 1982). In moths, a major class of female sex pheromone is unsaturated compounds of

10–18 carbons with oxygenated functional groups, mostly alcohols, their acetate esters or aldehydes (Bjostad et al., 1987; Arn et al., 1998).

There have been no previous reports of eicosanol and docosanol being involved in insect sexual communication. Although eicosanol was found in the sex pheromone blend of the grapevine moth *Lobesia botrana*, the actual pheromone components are unsaturated acetates of 12-carbon alcohols (Witzgall et al., 2005). Although volatile chemicals, which elicit electroantennogram responses, are released by *T. infestans,* this occurs during, and not before, copulation (Gonzales Audino et al., 2007; Crespo and Manrique, 2007). Our results show that mating attempts by a *T. infestans* male requires contact of the male's antennae with the female, and that epicuticular lipids trigger this response. Female-specific docosanol seems to be an important chemical that mediates this behavior. The characteristic triatomine aggregation behavior in small nests probably facilitates contact, and consequently recognition, of mates. Further studies on pheromone components may help the development of new control tools (Juárez et al., 2008; Pedrini et al., 2009) against Chagas disease vectors.

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Physiological Stress Responses, Fecal Marking Behavior, and Reproduction in Wild European Pine Martens (*Martes martes*)

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Abstract The relationship among physiological stress responses, fecal marking behavior, and reproduction in male and female European pine martens was investigated. Between July 2004 and June 2007, 145 fresh fecal samples were collected in a protected area of northwest Spain. Fecal DNA was used for specific identification by using the polymerase chain reaction-restriction fragment length polymorphism technique. Glucocorticoids (cortisol) and sex steroid hormones (P, progesterone; E, estradiol; T, testosterone) were quantified by enzyme immunoassays. Sex was assigned according to concentrations of T+P+E and the T/P ratio. Fecal cortisol concentrations were higher in males than in females. Feces with a presumptive marking function (on conspicuous substrates, above ground level, and/or in latrines) had higher mean levels of cortisol than those that were on inconspicuous substrates and/or at ground level, for both males and females. Fecal mark density was highest in spring, when mean levels of fecal cortisol were more elevated. Therefore, the higher physiological stress levels in females could be due to female physiological state (late-term pregnancy and lactation), competition for resources connected to birth, or food resources for offspring rearing. In males, the increase could be due to higher male competition for access to females during pro-estrus and estrus. Our results suggest that scent marking in European pine martens is related to reproduction and is involved in intersexual and intrasexual communication.

Key Words Feces · Glucocorticoids · *Martes martes* · Reproduction · Scent marking · Sex hormones

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Introduction

Chemical communication plays an important role in carnivore social and reproductive behavior. Many species use olfactory signals for information about reproductive state, territory defense, identity, group membership, and social status (Gorman and Trowbridge, 1989). Many species of mustelids use feces, urine, and the secretions of specialized glands as visual and scent marks for chemical communication (Pulliainen, 1982; Hutchings and White, 2000; Barja, 2005). In carnivores, the function of these marks is a matter of debate, but several studies have indicated that scent-marking behavior is related to reproduction, and is involved in basic functions such as defense of territory, intrasexual competition, and mate choice (Macdonald, 1985; Gosling and McKay, 1990; Gosling and Roberts, 2001). Scent marking in territories is best understood in terms of competitor assessment theory (Parker, 1974). Resource holders scent mark to provide opponents with a means of competitor assessment, thus reducing the costs of resource defense (Gosling and McKay, 1990).

Behavior can determine or modify the production and blood concentration of hormones (Mazur et al., 1992). However, little is known about the behavioral factors that cause an increase in glucocorticoid levels (e.g., scent marking, Barja et al., 2008a). When environmental, social, and physiological conditions are favorable, hormones coor-

dinate the physiological and behavioral changes associated with reproduction (Alcock, 1998). Sex steroid hormones (SSH) act on sexual organs, thus contributing to the development of reproductive processes, and are crucial in regulating patterns of reproductive behavior (Nelson, 2005). Studies on different carnivores indicate that concentrations of these hormones increase during the reproductive season (Velloso et al., 1998; Soto et al., 2004; Barja et al., 2007).

Cortisol is the primary glucocorticoid produced and secreted by the adrenal cortex. It is often referred to as a "stress hormone", as it is involved in the response to stress, affecting blood pressure, blood sugar levels, and other actions of stress adaptation (Axelrod and Reisine, 1984). Short-term glucocorticoid secretions have been related to adaptive responses of animals to stresses, and are considered beneficial for the organism (Möstl and Palme, 2002). An increase in glucocorticoid secretion is the front-line endocrine mechanism to defend an organism against stressful conditions. Additionally, glucocorticoids are released in response to situations not normally regarded as stressful, including courtship, copulation, and hunting. Moreover, during short-term stress, glucocorticoids improve fitness by mobilizing energy, and causing changes in behavior (Möstl and Palme, 2002). Nevertheless, prolonged periods of high cortisol levels can lead to pathologies (e.g., reproductive and/or endocrine disruption, immune suppression, and gastrointestinal ulcerations) that can reduce overall fitness (Dobson and Smith, 1995).

The quantification of glucocorticoids and SSHs in blood and urine requires capture and manipulation of animals which, in itself, can modify hormonal concentrations and lead to increases in physiological stress (Young et al., 2001). Quantification of these hormones in feces is a non-invasive method that is increasingly used in carnivore studies (e.g., Goymann et al., 2001; Soto et al., 2004; Creel, 2005; Barja et al., 2008a, b).

The European pine marten is an elusive, nocturnal carnivore that is difficult to study in the field. Relationships among glucocorticoid levels, fecal marking behavior, and SSH are not known for this mustelid. The only study on its reproductive behavior was performed using captive animals (Mead, 1994). The aims of the present study were: 1) to establish the relationship among fecal glucocorticoid levels, fecal marking frequencies and SSH concentrations in males and females of free-ranging European pine martens; and 2) to use these results to test current theories on the function of scent marking in territories.

Methods and Materials

Collection of Fecal Samples The study was conducted from July 2004 to June 2007 in Montes do Invernadeiro Natural Park, a mountainous area of 5,722 ha located in Northwest Spain. European pine martens use forest roads and deposit feces with visual and scent-marking functions (Pulliainen, 1982; Barja, 2005). To collect fresh fecal samples and study the fecal marking of this species, we surveyed forest roads, consisting of between 26 and 33 400 m-long transects, in 26 Universal Transverse Mercator cells of 1 km^2 each, in each season. The mean territory size of the European pine marten is 1.3 km^2 for males and 1.0 km^2 for females (O. Berdión, unpublished data). Thus, in order to increase the likelihood of obtaining fecal samples from different individuals and to minimize pseudoreplication, we collected from four zones of the study area, set far apart from one another and, in each zone, the 400 m transects were separated by a distance of 1 km. During the study we located 239 European pine marten scats, of which 145 were fresh. Of these 145, 90 were used in the DNA analyses and all for the hormonal analyses.

To quantify cortisol and SSH levels, samples of fresh feces were collected, from sunrise to 3 h after, thus ensuring that exposure time to environmental conditions and microbial action was minimized (Millspaugh and Washburn, 2004). We collected all samples from the fine end of the scat to standardize sampling. Feces were considered fresh when they had a layer of mucus, a high level of hydration, and a strong odor. From each fresh scat, 10 g of sample were gathered and the date recorded. In the field, the fecal samples were placed in a portable refrigerator loaded with ice. Because freezing of such samples is considered the best method of preserving hormone content (Millspaugh and Washburn, 2004), all samples were frozen within 5 h of collection and maintained at −20°C until assayed.

Specific Identification of Feces Based on morphology, pine marten feces are difficult to distinguish from those of medium-size carnivores such as red fox (*Vulpes vulpes*), wildcat (*Felis silvestris*), and stone marten (*Martes foina*) (Davison et al., 2002). Therefore, we analyzed fecal DNA, from the first 90 fresh samples collected, by polymerase chain reaction–restriction fragment length polymorphism analysis (Ruiz-González et al., 2008). This showed that 80 of the samples were from European pine marten. The remaining ten samples were not amplified by PCR. The DNA results were consistent with our morphological identification of feces in the field. Therefore, the remaining (55) samples used in this study were identified by morphological characteristics.

Fecal Marking For fecal marking behavior, the following information was recorded for feces found in transects: "conspicuousness of substrate", height of substrate, and location in latrines (Barja, 2005). In order to analyze the

variable conspicuousness of substrate, we established two categories: inconspicuous and conspicuous. Conspicuous substrates were those that, for a human observer, stood out from the surrounding substrates (e.g., mound of soil, plants, stones). All others were regarded as inconspicuous. When a scat was on the ground, but less than 10 cm from a conspicuous substrate, we surveyed the substrate to check whether there were remains of scat or not; in this case, we considered that scat had a presumptive marking function. For the variable height, feces were considered as being above ground level or at ground level. We considered feces to be in latrines when two or more scats were concentrated at a defecation point. Feces were assumed to have a presumptive scent-marking function only when they were placed on a conspicuous substrate, above ground level, and/or in latrines. However, we do not exclude the possibility of scats placed on the ground and out of a latrine also serving a marking function.

Quantification of Glucocorticoids and Sex Steroid Hormones Fecal samples were analyzed for levels of cortisol, testosterone (T), progesterone (P), and estradiol (E). Hormones were extracted using methods for similar carnivore species (Brown et al., 1994) and for European pine martens (Barja et al., 2007). The efficiency of extraction was tested by the addition of radiolabeled hormones (^{3}H-cortisol, ^{3}H-testosterone, ^{3}H-progesterone, and ^{3}H-estradiol; 4,000–8,000 dpm; ICN, California, USA) prior to extraction of a parallel set of fecal samples.

SSH and cortisol concentrations were determined from fecal extracts by an enzyme immunoassay developed in our laboratory, and previously validated for the European pine marten (see assay protocol in Barja et al., 2007). Hormone concentrations were calculated by means of software developed for this technique (ELISA-AID, Eurogenetic, Belgium). Standard-dose response curves were constructed by plotting the binding percentage (B/B×100) against the standard hormone concentrations added.

The mean recovery of ^{3}H-steroids from fecal extracts was as follows: ^{3}H-cortisol, 81.5%; ^{3}H-testosterone, 85.4%; ^{3}H-progesterone, 85.5%; and ^{3}H-estradiol, 92.8%. Parallel displacement curves were obtained for each hormone by comparing serial dilutions of pooled fecal extracts and standard curves. The results showed that both curves were parallel in all cases. Intra- and inter-assay coefficients of variation were calculated by assaying 10 replicates of a pooled fecal sample in the same assay and 10 replicates of the same sample in 10 consecutive assays. The percentages recovered were, respectively, as follows: cortisol, 6.4 and 9.5%; testosterone, 5.5 and 8.9%; progesterone, 4.5 and 6.9%; estradiol, 4.9 and 7.5%. For each sample, SSH and cortisol concentrations are expressed in ng/g of dry feces.

Origin of the Fecal Samples Previous studies on carnivores showed that the concentration of T+P+E and the relation of T/P were higher in males than in females (Velloso et al., 1998; Soto et al., 2004). Therefore, to assign sex to the depositors of particular fecal samples, we carried out a k-means conglomerate analysis, using the concentrations of T+P+E and the T/P ratio of each sample (Barja et al., 2008b). This analysis pooled the samples in groups, or conglomerates, so that each group was as homogeneous as possible, and different from the other groups, for the variables analyzed.

Data Analysis We used one-way ANOVA, with year as a fixed factor and cortisol levels as a response variable. We used two-way ANOVA to test whether cortisol levels vary in relation to two factors: sex and fecal marking. The differences in cortisol concentrations between seasons were analyzed using one-way ANOVA. We used Games-Howell tests for *post-hoc* testing. A χ^2 test was used to verify if there were seasonal differences in the number of deposited feces with a presumptive marking function or not. For seasonal analysis, the months were grouped as follows: spring (April–June), summer (July–September), autumn (October–December), and winter (January–March). We consider this grouping appropriate because the months in each season have temperatures similar to each other in the study area. The relationship between seasonal fecal marking frequencies and SSH levels was analyzed using Spearman's correlation coefficient. Pearson's correlation coefficient also was used to determine the relationship between concentrations of fecal glucocorticoids and SSH. Fecal sex hormone and cortisol concentrations are given as mean ± SE. A p value$\leq$0.05 was considered significant. All analyses were carried out using SPSS version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Sex Assignment in Relation to Sex Hormone Concentrations Hormone concentrations (cortisol, T, P, and E) were analyzed for 145 fresh fecal samples of European pine marten. The k-means conglomerate analysis for the variables T+P+E and T/P revealed that the samples belonged to two different groups. Group 1 comprised the greater number of samples (83.4%) and had concentrations of T+P+E of 133.9±13.6 ng/g of dry feces (range to 697.61) and a T/P ratio of 0.30±0.03 ng/g of dry feces (range to 2.49) that were lower than those of group 2. Group 2 comprised 16.6% of the fecal samples, all with a high concentration of T+P+E (1399.0±97.1 ng/g of dry feces) and a high T/P ratio (0.90±0.1 ng/g of dry

feces; range 3.28). The differences between both groups for T+P+E and T/P were significant (T+P+E: ANOVA, $F_{1,\ 145}$= 580.23, P<0.001; T/P: ANOVA, $F_{1,\ 145}$=29.12, P<0.001). Therefore, the fecal samples of group 1 belonged to females and those of group 2 to males (Velloso et al., 1998; Soto et al., 2004).

Fecal Glucocorticoid Levels and Fecal Marking The differences in fecal cortisol levels between years were not significant (one-way ANOVA: $F_{4,145}$=0.109, P=0.955). Therefore, the results of both years were pooled for further statistical analysis. The fecal cortisol levels in European pine marten were dependent on sex (males, 58.9±10.1 ng/g of dry feces; females: 22.6±4.9 ng/g of dry feces) (two-way ANOVA: $F_{1,\ 145}$=46.6, P=0.014) and fecal marking (with a presumptive marking function, 36.4±10.6 ng/g of dry feces; no presumptive marking function, 25.7±5.0 ng/g of dry feces) (two-way ANOVA: $F_{1,\ 145}$=9.72, P=0.051). The interaction between fecal marking and sex was not significant (two-way ANOVA: $F_{1,\ 145}$=3.43, P=0.187). In males and females, cortisol levels were higher in feces that had a presumptive marking function than those that did not. In both feces with a presumptive marking function and those with no presumptive marking function, males had higher cortisol levels higher than females (Fig. 1).

Seasonal Variation in Fecal Marking Frequencies and Sex Hormone Levels The spatial location of 239 scats was categorized for fecal marking. A presumptive marking function was evident in 23.4% of scats, as they were deposited on conspicuous substrates, above ground level, and/or in latrines. Density of feces with a presumptive marking function was highest in spring and lowest in winter. Fecal marking densities were similar in summer and autumn (Fig. 2). There were seasonal differences in fecal marking frequencies (χ_3^2=30.54, P<0.001). Mean fecal glucocorticoid levels were higher in spring than in summer, winter, and autumn (one-way ANOVA: $F_{3,\ 145}$=2.30, P= 0.080; *post-hoc* Games-Howell test: P=0.025 for spring and autumn, and P>0.05 for comparisons among the other seasons; Fig. 2).

We found a positive correlation between fecal testosterone concentrations and seasonal fecal marking (Spearman correlation: r=0.223, N=4, P=0.007). However, the concentrations of progesterone (Spearman correlation: r=0.0911, N=4, P=0 .275) and estradiol (Spearman correlation: r=0.064, N= 4, P=0.444) were not associated with fecal marking frequency. In addition, fecal cortisol levels were positively associated with the three SSHs examined, as follows: T (Pearson correlation: r=0.372, N=145, P<0.001), P (Pearson correlation: r=0.544, N=145, P<0.001), and E (Pearson correlation: r=0.564, N=145, P<0.001).

Discussion

In the present study, the highest density of feces with an evident marking function occurred in spring; these spring feces also had the highest concentrations of fecal cortisol. This result is consistent with a previous study in the same study area, which showed that fecal concentrations of T, P, and E were higher in spring than in other seasons (Barja et al., 2007). Given that scent marking is important for mustelids in sexual communication (Pulliainen, 1982; Hutchings and White, 2000; Barja, 2005), and that SSHs regulate sexual behavior in mammals (Velloso et al., 1998; Nelson, 2005), it appears that SSHs may play a key role in modulating fecal marking behavior in mustelids, such as the European pine marten. The low density of feces with a presumptive marking function in autumn coincided with the non-reproductive period (delayed implantation) of the

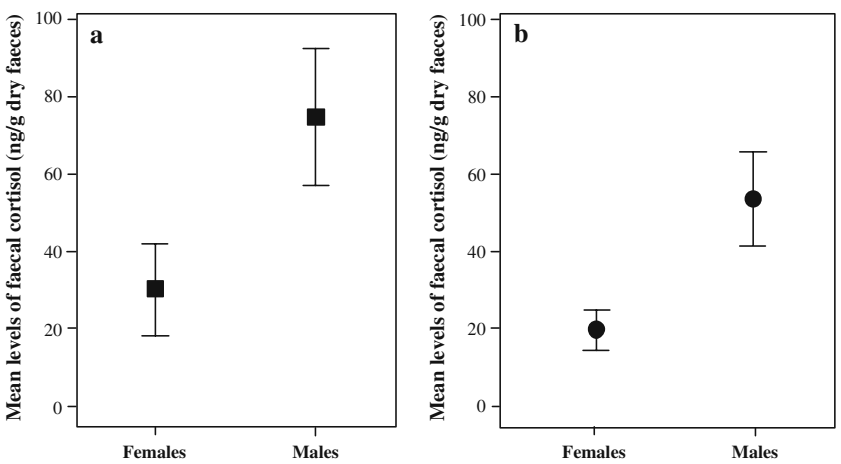


Fig. 1 Comparison of fecal cortisol levels (mean ± SE) of male and female European pine marten, according to presumptive fecal marking. **a** Feces with a presumptive marking function, and **b** feces with no presumptive marking function

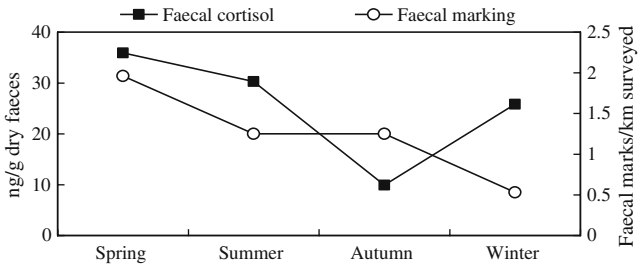


Fig. 2 Seasonal variation in the density of European pine marten feces with a presumptive marking function, and fecal cortisol levels

species (Mead, 1994). This is consistent with European pine martens having low SSH levels in this season (Barja et al., 2007).

In our study, the increase in fecal cortisol levels and in density of feces with a presumptive marking function during the spring and, to a lesser extent, in summer (when births and estrus occur; Mead, 1994), likely reflects the sex-biased cost of reproduction. Short-term activation of adrenocortical hormone secretion, associated with a low degree of physiological stress, seems to be beneficial for reproduction by maintaining the animal's level of responsiveness to socio-sexual stimuli necessary for sexual arousal and reproductive activation (Carlstead and Shepherdson, 1994). However, long-term elevation of glucocorticoid levels can disrupt reproductive behavior and the reproductive hormone axis, causing decreases in an animal's chances of finding mates, initiating pair formation, and copulating, as well as possibly arresting ovulation (Pierce et al., 2008). We do not know whether or not the fecal cortisol levels observed in this study are indicative of decreased fitness.

Females could be maximally stressed in spring by their physiological state (i.e., late-term pregnancy and lactation; Mead, 1994), as reported in other mammals, such as the ring-tailed lemur (Starling et al., 2010). This would indicate that female martens experience increasing physiological costs as pregnancy progresses and throughout the period of exclusive infant nursing. In addition, the increase in cortisol levels in females could be due to intrasexual competition for resources connected to birth (nest sites) and food resources for offspring rearing. Changes in glucocorticoid levels also have been linked to food availability in wild olive baboons (Sapolsky, 1986). In males, the highest physiological stress levels may be due to territory defense and to intrasexual competition over females during pro-estrus and estrus. Scent marks may allow intruders to assess the identity of the territory owner, and thus avoid the high cost of territory defense (Gosling and McKay, 1990). Studies should be carried out to confirm this, because the sample size of males in our study was small.

In our study population, cortisol levels were higher in feces that had a presumptive marking function than in those that did not, both for males and females. Similar results have been observed in other carnivores, such as the Iberian wolf (Barja et al., 2008a). Higher cortisol levels in prominent fecal marks support the idea of scent marking as part of competition between males and females. The costs that lead to higher stress are likely to be costs of competition (including injury), which a signaler risks when its marks are more conspicuous to competitors. Scent marks also may be condition-dependent signals of quality and competitive ability, thus providing a means of assessment about the signaler's quality (Roberts and Gosling, 2001). Competitors could identify resource holders by investigating fecal scent marks from a given area, and match these with the owner's odor when they meet, a mechanism known as the scent-matching hypothesis (Gosling, 1982). European pine martens are solitary animals that are territorial toward members of their own sex. The territory of a male often overlaps extensively with those of several females (Macdonald, 2001). Territories within a sex can overlap, but every individual uses parts of the shared area exclusively (Blanco, 1998). As the chance of invading occupied locations in these overlapping areas is probably higher than in a strict territorial system, scent matching could modulate intrasexual aggression among females and males in non-exclusive areas, as reported for male snow voles (Luque-Larena et al., 2001).

Testosterone concentrations were higher when presumptive fecal marking was more frequent, suggesting that this is associated with aggressiveness of males and the defense of territories. When females are in estrus, there is intense competition between males for territories that determine priority of access to mates (Pulliainen, 1982; Mead, 1994; Schroepfer et al., 1997). Several studies indicate that testosterone plays a key role in the modulation of aggression (Lincoln et al., 1972; Alcock, 1998) and that in mammals males have higher levels of testosterone and are more aggressive than females (Lincoln et al., 1972; Barja et al., 2008b). Higher levels of aggressive behavior are associated with estrus and with defense of territory in ungulates and primates (Bouissou, 1983). Therefore, our results indicate that fecal testosterone in European pine martens may play a key role in the regulation and modulation of fecal marking by males in relation to reproduction and intrasexual competition.

Quantification of fecal steroid hormone levels is a useful method for detecting and monitoring reproduction in threatened and endangered carnivores, because quantification of fecal hormone levels is a non-invasive method for evaluating gonadal function (Young et al., 2004). In addition, fecal hormone quantification avoids additional stresses that can be caused by animal capture, handling, sedation and/or transport (Brown and Wildt, 1997). A better understanding of carnivore reproductive physiology, in conjunction with

behavioral studies, could facilitate breeding, management, and conservation activities, of endangered species.

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RAPID COMMUNICATION

The Effect of Elevated Steroids Released by Reproductive Male Round Gobies, *Neogobius melanostomus*, on Olfactory Responses in Females

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Abstract The round goby, *Neogobius melanostomus*, is a highly successful invasive species in the Laurentian Great Lakes. Previous behavioral studies implied that females are attracted by pheromones to the nests of reproductive males, and that males release putative steroidal pheromones—unconjugated as well as conjugated forms of 3α-hydroxy-5β-androstane-11,17-dione (11-O-ETIO)—following stimulation of the hypothalamic-gonadal axis with salmon gonadotropin releasing hormone analog (sGnRHa). In this study, we tested the olfactory system of females in response to extracts containing these released steroids. We compared electrical field potential responses from the olfactory epithelium (electro-olfactogram, EOG) of non-reproductive females to methanol extracts of water that previously held males, collected before and after injection of the males with sGnRHa or saline. The females showed increased EOG responses to the post-injection extracts when males were treated with sGnRHa but not saline. This finding provides further evidence for interactions between male and female *N. melanostomus* via steroidal reproductive pheromones.

Key Words Electro-olfactogram (EOG) · Gonadotropin releasing hormone · Hormone · *Neogobius melanostomus* · 11-Oxo-etiocholanolone · Pheromone · Round goby · Steroid

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Introduction

Many fishes use hormonal products released into the water as reproductive pheromones (Stacey et al., 2003). Gonadotropin releasing hormone (GnRH) is an important factor in the production and release of steroidal hormones, as it stimulates the release of luteinizing hormone, which is responsible for stimulating gonadal steroid release (reviewed by Zohar et al., 2010). GnRH is known to increase plasma steroid levels, but it is less clear whether GnRH can lead to an altered chemical signal, or whether this signal could in turn affect the response of opposite-sex conspecifics. Serrano et al. (2008) showed that treating male peacock blennies, *Salaria pavo*, with 11-ketotestosterone increases female olfactory responses to extracts of the gonads and anal glands. The olfactory system of the round goby, *Neogobius melanostomus* Pallas, responds to steroidal compounds (Murphy et al., 2001), and reproductive females show increased olfactory and behavioral responses to water from reproductive males vs. non-reproductive males (Belanger et al., 2004; Gammon et al., 2005). Katare et al. (2011) observed that following injection with salmon GnRH analog (sGnRHa), male *N. melanostomus* increase the release of several steroids into the water, including 11-oxo-etiocholanolone (11-O-ETIO) and 11-O-ETIO-3-sulfate (11-O-ETIO-3-s), which are novel fish steroids and putative pheromones in this species. We used electro-olfactogram (EOG) recordings of female *N. melanostomus* to determine how a surge of sGnRHa would affect the olfactory signal released by males.

Methods and Materials

Experimental Animals Neogobius melanostomus were angled from the Detroit River and Lake Erie in Windsor and

Leamington, Ontario, respectively. Fish were separated by sex and held in the animal facilities at the University of Windsor in flow-through aquaria with dechlorinated municipal tap water at ambient temperatures (~20°C) on a 16:8 h photoperiod. Fish were fed commercial food once daily (Aquatic Eco-systems, Apopka, FL, USA). All experimental procedures conformed to guidelines of the Canadian Council for Animal Care.

Generation of Methanol Extracted Male Water The procedure for generating methanol extracts of water that held reproductive males (RM) has been described elsewhere (Katare et al., 2011). Each RM (chosen on the basis of secondary sexual characters; Belanger et al. 2004) was isolated in 1 l of dechlorinated water, which was collected after 4 h ("pre-injection"). The RMs were given an intramuscular injection of either 20 μg/kg sGnRHa (Ova-RH, Syndel Laboratories, Qualicum Beach, BC, Canada; N=6) dissolved in 0.9% saline at 4 μg/ml, or 0. 9% saline (N=3). RMs then were immersed into fresh dechlorinated water (1 l) for 16 h, and this water was collected ("post-injection"). Steroids were extracted from the water (1 l) by passing it through activated, rinsed C18 cartridges (Sep-Pak, Waters, Milford, MA, USA) and eluting with 5 ml of methanol, a 200-fold concentration. C18 cartridges were used only once and then discarded. Stock solutions of methanol extracted steroids were produced by diluting 100 μl of extract into 10 ml of dechlorinated water (a 100-fold dilution). Individual stocks were diluted 100× further in dechlorinated water for EOG testing; thus, tested concentrations of extracted compounds were all below the original concentrations produced by the RMs. We used a custom immunoassay to measure the amount of released immunoreactive unconjugated and conjugated 11-O-ETIO in the methanol extracts (Katare et al., 2011). The release rate of immunoreactive 11-O-ETIO was 2.13±0.64 ng/ml/h in methanol extracts prepared prior to injection. The release rate was 0.95±0.57 ng/ml/h (N=3) for saline-treated RMs and 8.08±2.12 ng/ml/h for sGnRH-treated fish (N=6).

Electro-olfactogram (EOG) Recordings We recorded summed generator potentials from the surface of the olfactory epithelium by using a recording procedure adapted from Murphy et al. (2001) and Belanger et al. (2004). Non-reproductive female *N. melanostomus* were used for all recordings (N=7). Fish were anesthetized by submersion in a bath containing tricaine methanesulfonate (MS-222; Finquel®, Argent Chemical Laboratories, Redmond, WA, USA) at 100 mg/l. Dechlorinated municipal tap water (used as background water) was dripped constantly over the olfactory epithelium via a tube positioned over the posterior naris (nostril). Odors were introduced into this water flow via a solenoid-driven switching mechanism to avoid mechanical stimulation. The standard odor, 10^{-5} M L-alanine (Sigma-Aldrich, Oakville, ON, Canada), was delivered periodically to monitor the stability of the recording. We recorded responses to pre- and post-injection extracts. EOG responses were recorded in millivolts (mV) and normalized by dividing them by the mean response to L-alanine. When more than one female was exposed to the methanol extract of a particular male, those females' responses were averaged together to obtain one mean response value for each individual extract. We used a data acquisition system (PowerLab, ADInstruments, Colorado Springs, CO, USA) and computer to record and analyze the data. Data were analyzed with SigmaStat 3.5 (Systat, San Jose, CA, USA) by using a paired t-test to analyze for differences in EOG response to pre-injection vs. post-injection extracts (from sGnRHa- and saline-treated fish).

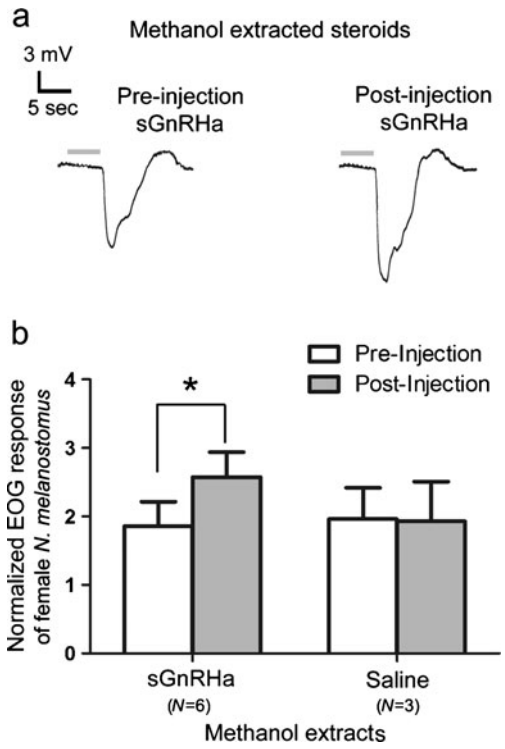


Fig. 1 Electro-olfactogram (EOG) responses to methanol-extracted conditioned water from reproductive male (RM) *Neogobius melanostomus* before and after injection with either sGnRHa or saline. **a** Typical EOG trace from female *N. melanostomus* to pre-injection and post-injection (with sGnRHa) methanol extracts from an RM; **b** female *N. melanostomus* show an increased and significant (*, P= 0.02) EOG response to the post-injection methanol extract of conditioned water when the RM was treated with sGnRHa, but not when it was treated with saline

Results

Methanol extracts of RM *N. melanostomus*-conditioned water elicited robust olfactory responses from female *N. melanostomus* (Fig. 1a). Females had a greater EOG response to the methanol extracts following treatment of males with sGnRHa (t=3.23, P=0.02, df=6); the response did not differ following treatment of males with saline (t= 0.24, P= 0.83, df= 2; Fig. 1b).

Discussion

We observed that olfactory responses by female *N. melanostomus* to odors from the male were greater after the male had been treated with sGnRHa—a treatment known to increase the male's release of putative pheromones (Katare et al., 2011). This finding indicates that females may be attuned to, and subsequently stimulated by, endocrine changes in the male through release of chemical signals, and it provides additional evidence for the use of sex pheromones by *N. melanostomus*. While Belanger et al. (2004) observed an increased EOG response from reproductive female *N. melanostomus* to water that held RMs, our findings indicate that even non reproductive females are capable of responding to changes in the endocrine status of the male, and that compounds that elicit this olfactory response have the properties of steroids. Although immunoreactive 11-O-ETIO was quantified in the RM water extracts (Katare et al., 2011), other steroids also captured by the C18 columns may have contributed to the increased olfactory responses. This increased olfactory response may translate to behavioral effects in the female, as responses to urine from sGnRHa-treated males have been observed (Kereliuk, 2009). In order to synchronize spawning between the sexes, many fish species have evolved the use of hormonal products as pheromones, as these signals are indicative of the reproductive status of the sender. In goldfish, *Carassius auratus* (with the best characterized reproductive system involving identified pheromones), females experience a surge in gonadotropin II in response to environmental factors, which in turn induces the females to release 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) (Stacey et al., 1989). Release of 17,20β-P in turn stimulates a gonadotropin increase in the males (Stacey et al., 1989), and leads to the initiation of reproductive behavior (DeFraipont and Sorensen, 1993). Thus, increasing the titer of GnRH is the necessary first step leading to spawning in both male and female *C. auratus*.

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RAPID COMMUNICATION

Low Concentrations of Salicylic Acid Stimulate Insect Elicitor Responses in *Zea mays* Seedlings

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Abstract Salicylic acid (SA) generally is thought to suppress jasmonic acid (JA) related signaling events. However, when we treated the roots of corn seedlings overnight with low physiological concentrations of SA (50 μM), we found a priming effect of this pretreatment on typical insect elicitor (IE)-induced responses in the leaves of these plants. IE-induced JA was more than 2-fold up regulated in SA-pretreated plants. Consequently, IE-induced volatile organic compounds (VOC) release also was significantly increased. In contrast, when corn seedlings were treated with SA overnight and then mechanically damaged, we found no significant differences in JA accumulation. We also found that the application of even lower concentrations of SA (5 μM) had no significant effect on IE-induced responses, while higher concentrations (500 μM) inhibited IE-induced JA accumulation. Likewise, shorter exposure to SA did not affect subsequent JA accumulation induced by IE or mechanical wounding. These results provide evidence for the existence of non-compatible defense priming by signaling molecules that usually are involved in a conflictive defense signaling pathway and suggests common elements in the regulation of priming plant defense responses.

Key Words Jasmonic acid · Salicylic acid · Insect elicitors · *N*-linolenoyl-glutamine · Priming · Volatiles

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Introduction

In the plant defense responses to pests and pathogens, two major hormones are responsible for regulating specific countermeasures, salicylic acid (SA) and jasmonic acid (JA). Salicylic acid is an important signal in the plant response against pathogen or viral infections, while JA is the major regulator in insect-herbivore response, including the production of proteinase inhibitors, toxic secondary metabolites, and the production and release of volatile organic compounds (VOC). The latter may serve as important signals in tritrophic interactions by attracting predators and parasites of attacking insect herbivores and they have been demonstrated to be quite effective (Kessler and Baldwin, 2001). Besides causing significant mechanical damage to the plant, insect herbivores also apply compounds abundant in their saliva to the wounding site. These elicitors often potentiate the plant's defense responses by causing a significant higher accumulation of JA and producing more VOC, a defensive measure when compared to mechanically damaged control plants (Schmelz et al., 2009).

The interaction of the SA- and JA-pathway in this context is of particular interest due to the fact that often activation of one pathway inhibits the other. Examples for this interaction have been described frequently (Koornneef et al., 2008 and refs. therein). However, other reports indicate that SA also may have a stimulatory effect on certain JA-mediated responses. Mur et al. (2006) reported that this interaction depends on the concentration of the hormones involved, and found that lower concentrations of SA, when applied concomitantly with JA, caused a significant increase in certain JA-mediated responses. Similarly, elicitors like alamethicin and inceptin, and also

certain pathogens, activate both signaling pathways concomitantly in corn, cowpea, *Arabidopsis*, and tobacco, respectively (van Wees et al., 2000; Engelberth et al., 2003; Schmelz et al., 2009; Mur et al., 2006). It seemed that in those cases SA did not have any inhibitory effects. High concentrations (500 μM–1 mM) of SA also were used to test for effects of this compound on JA levels and JA-mediated processes. However, when endogenous levels of SA in corn after treatment with alamethicin were calculated, the average concentrations in those plants were much lower (Engelberth et al., 2003). Therefore, we decided to use these lower, more physiological amounts to test for the effects of SA on induced JA accumulation. We found that treatment with a low concentration of SA significantly increased IE-induced JA production and VOC release when applied to the root system of the plant. To our knowledge, this is the first report on a priming effect of SA on insect herbivore-related and JA-mediated defense responses in plants.

Methods and Material

Salicylic acid was purchased from Sigma chemicals (St. Louis, MO, USA). Corn [*Zea mays* cv. Kandy Korn; Jung Seed Company (www.jungseed.com)] was grown in plastic pots (size appr. 10×10×8 cm) filled with approximately 400 cm^3 soil (Ready Earth Plug & Seed, SunGro, MA, USA) at 26°C, 12:12 h, L:D conditions. Light intensity was set at app. 150 μmol m^2 s^{-1}.

SA was applied at a 5, 50, or 500 μM by watering 14-d-old corn seedlings (V2 stage, 3rd leaf already well developed) with 100 ml of the respective solution in water/methanol (99:1 vol/vol) per pot overnight for 15 h prior to treatment with insect elicitor (IE; here: *N*-linolenoyl-glutamine) or with mechanical wounding (MW). Controls were treated with the water/methanol mixture alone. IE was applied as described previously (Engelberth et al., 2004). In brief, an area of about 2×10 mm on the second leaf of each plant was scratched with a razor blade and 10 μl of the IE solution (100 pmol/μl in 50 mM KPi buffer, pH 8) immediately added to the wounded site. Mechanical wounding was done similarly without adding buffer. Leaf segments (about 2.5 cm in length) comprising the IE or MW application site were cut 1 and 2 h after treatment, and analyzed for JA and SA accumulation. Control segments without damage were taken from SA- and water-treated plants.

Jasmonic acid and SA were extracted and quantified by vapor phase extraction (Engelberth et al., 2004). In brief, frozen plant tissues were extracted with a mixture of 1-propanol:H_20:HCl (2:1:0.002) and dichloromethane. After phase separation, the organic phase was transferred and evaporated under a constant air stream, re-dissolved in 100 μl of diethyl ether: methanol (9:1, vol:vol), and subsequently methylated by diazomethane. Thus, volatilized metabolites were separated from the complex mixture by vapor phase extraction and analyzed by GC/MS.

To test for the effects of SA on IE-induced VOC, intact corn plants were incubated with SA overnight. The next morning, plants were treated with IE, and 5 h later were cut at the root base, wrapped in wet tissue paper, and transferred to 200 ml glass cylinders, where VOC were collected for 1 h as described (Engelberth et al., 2004), and analyzed by GC/MS with 3-octen-2-one as internal standard. At least three biological replicates of all experiments were

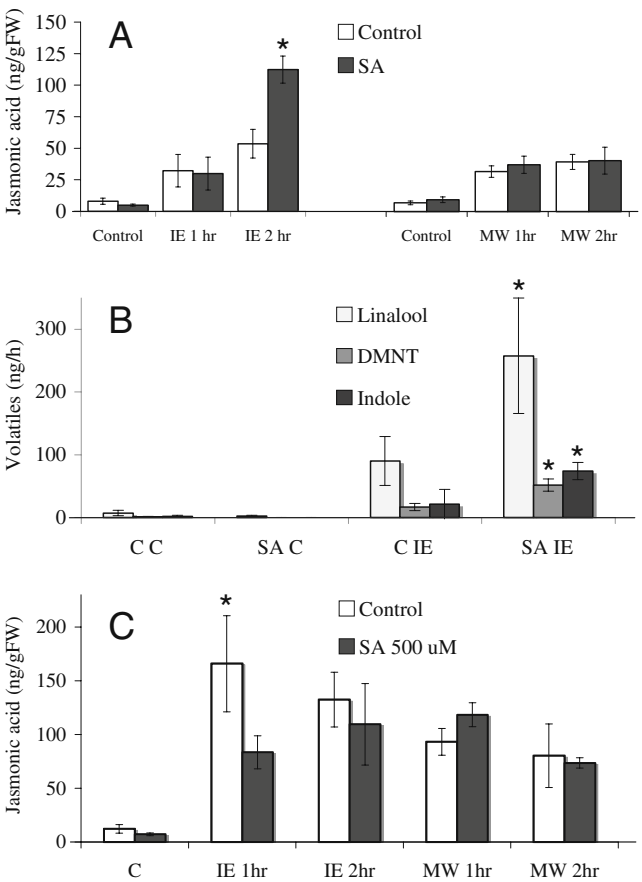


Fig. 1 Effects of salicylic acid (SA) on wound (MW)- and insect elicitor (IE)-induced total jasmonic acid accumulation and volatile release in *Zea mays*. **a**: Jasmonic acid accumulation measured 60 and 120 min after treatment with insect elicitors or mechanical wounding in SA pretreated and control plants. **b**: Volatile release of SA-pretreated and control plants 5 hr after induction with insect elicitors. For both experiments (**a** and **b**), roots of corn seedling were watered with 100 ml of a 50 μM SA aqueous solution overnight (15 hr). DMNT, (*3E*)-4,8-dimethyl-1,3,7-nonatriene; C C, untreated control; SA C, SA pretreated control. **c**: A higher concentration of SA (500 μM) inhibits IE-induced JA accumulation at 1 h. A *t*-test was used to test for significance (*, $P \leq 0.05$) compared to the respective control. Data represent mean ± standard error ($n \geq 3$)

performed. Data were analyzed for significance with *t*-test ($P<0.05$). Treatments were compared to appropriate controls.

Results and Discussion

We tested SA for its effect on IE- and MW-induced JA accumulation and VOC release. When roots of corn seedlings were pretreated with 50 μM SA for 15 h and then the leaves induced by application of IE, we found a significant increase in JA production (112.4 ng/g FW) compared to IE-induced control plants (53.5 ng/g FW) 2 h after IE application. Mechanical wound-induced JA was not affected in SA treated plants (Fig. 1a). We also did not detect any increases in free or conjugated SA over control plants (data not shown). While even lower (5 μM) concentrations of SA had no significant effect on MW- or IE-induced JA accumulation (data not shown), we observed a significant reduction in IE-induced JA accumulation 1 h after elicitation in plants treated with 500 μM SA (Fig. 1c). Shorter treatment periods (4 and 8 h) with SA had no effects on JA accumulation (data not shown).

Consequently, 15 h SA pre-treated plants produced significantly more VOC when induced by IE (Fig. 1b). Most affected were linalool, (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and indole, while no increases in sesquiterpenes were observed. Overall, a 3-fold increase for combined volatiles was found for SA-pretreated plants. It is noted that no MeSA emission as a consequence of the pre-treatment of corn seedling with SA was observed.

The priming effect of SA on JA and VOC release after induction with IE in *Zea mays*, as described herein, bears close resemblance to those observed for corn plants exposed to green leaf volatiles (GLV) (Engelberth et al., 2004). When corn seedlings were exposed to physiological concentrations of GLV overnight and subsequently induced with IE, they also exhibited a stronger response with regard to JA accumulation and VOC release when compared to appropriate controls. As for SA treatment, MW-induced JA accumulation was not affected.

Defense "priming" describes a process in which a response to a certain challenge (i.e., pathogen infection or herbivore infestation) is accelerated and/or enhanced by challenge-specific prior stimulation. A requirement for priming is for the plant to be either previously challenged by either nonpathogenic strains of potential pathogens, or treated with compatible chemicals or analogs of those compatible chemicals that are usually also involved in direct defense signaling (Prime-A-Plant Group, 2006; Frost et al., 2008). For example, SA as a major direct defense signal primes defense responses against pathogen attack at low concentrations. Likewise, priming against insect herbivory is mostly signaled by methyljasmonate and/or green leaf volatiles (GLV), compounds that are also involved in direct anti-herbivore defense signaling. However, the data presented herein are in contrast to those forms of compatible priming. Corn seedlings appear to be capable of utilizing signals from a conflicting pathway to prime anti-herbivore defenses, when those signals are recognized at appropriate concentrations. Mur et al. (2006) described a comparable stimulatory effect of SA on typical JA responses. However, both compounds were applied exogenously, and no treatment was tested for its effect on endogenously induced responses, for example JA accumulation after mechanical wounding or pathogen infection. In our study, only SA was applied exogenously, while JA accumulation was induced *in planta* by treatment with IE and MW. Furthermore, SA exhibited its priming effects only when applied for 15 h, and was specific towards IE-induced JA accumulation, whereas MW-induced JA was not affected. It is well known that mechanical damage and insect herbivory exhibit certain differences, which can be attributed to IE like volicitin, *N*-linolenoyl-glutamine, and other fatty acid-amino acid conjugates abundant in the spit of the attacking insect herbivore (Engelberth et al., 2007; Schmelz et al., 2009). In corn, major differences between MW and IE application were found with regard to the spatial distribution of major jasmonates (Engelberth et al., 2007), and it appears that this context-dependent accumulation of jasmonates is a critical factor for the initiation and effectiveness of GLV- and SA-induced anti herbivore defense priming. The similarities between GLV- and SA-induced priming also further suggest that common mechanisms may exist, which govern the primed state of the plant. Work is currently in progress to identify elements that regulate this response.

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Effects of Time After Last Herbivory on the Attraction of Corn Plants Infested with Common Arymworms to a Parasitic Wasp *Cotesia kariyai*

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Abstract Females of the gregarious endoparasitoid *Cotesia kariyai* were attracted to a blend of volatiles released from corn plants infested with larvae of their host, the common armyworm (*Mythimna separata*). We investigated the effects of time after the last infestation (1–168 h) on the attractiveness of corn plants infested by host larvae by using a wind tunnel under laboratory conditions. Immediately after the removal of the larvae, parasitoids were attracted more to plants that had been infested with the larvae than to intact plants (control). This attractiveness gradually decreased with time after the last infestation. The attractiveness of the infested plants was significantly higher than that of intact plants when the time after the last infestation was within 1 day. Fifteen herbivore-induced volatiles were recorded in the headspace of infested corn plants irrespective of time. The amounts of some compounds including (*Z*)-3-hexen-1-yl acetate, which have already been reported to attract *C. kariyai*, correlated with the attractiveness. The ecological meaning of the duration of production of *C. kariyai* attractants is discussed.

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Key Words Herbivore-induced plant volatiles · *Mythimna separata* · *Cotesia kariyai* · Flight response

Introduction

Plants respond to herbivore attack and defend themselves against herbivores either directly or indirectly (Karban and Baldwin, 1997; Arimura et al., 2009). These plant responses are called induced direct defenses and induced indirect defenses. For induced direct defense, plants produce chemical weapons such as toxins, repellents, and digestibility reducers that target the herbivores preference and performance (Karban and Baldwin, 1997). One of the well known induced indirect defenses is the attraction of carnivorous natural enemies of the herbivores by emission of herbivore induced plant volatiles (HIPVs) (Takabayashi and Dicke, 1996; Arimura et al., 2009).

Mythimna separata (Walker) (Lepidoptera: Noctuidae) is a pest of various gramineous crops in Australia, New Zealand, and the tropic and temperate zones of Asia (Kanda, 1991). One dominant natural enemy of *M. separata* larvae is *Cotesia kariyai* Watanabe (Hymenoptera: Braconidae) (Hirai, 1984), a gregarious endoparasitoid. Females of *C. kariyai* prefer to oviposit in the 3rd to early 6th larval instar of *M. separata* (Sato and Tanaka, 1984). Takabayashi et al. (1995) reported that *C. kariyai* was attracted to HIPVs emitted from corn plants infested by early instars of *M. separata* larvae. The flight response of *C. kariyai* also was affected by host plant species, leaf age, and wasp experience (Fujiwara et al., 2000a, b; Fukushima et al., 2001; Hou et al., 2005).

From the viewpoint of carnivorous natural enemies, HIPVs emitted by herbivore-infested plants are useful

information for indicating the presence of host/prey (Vet and Dicke, 1992). Thus, HIPVs released by plants also are expected to influence the fitness of natural enemies. It would be adaptive for carnivores to find a plant that is currently infested by enough suitable prey/hosts. However, in a field, there are host/prey-infested plants with various histories of herbivory (i.e., number of host/prey, their developmental stages, time after the last infestation, etc.). Until recently, most chemical and behavioral studies on HIPVs have been on plants currently infested by herbivores or plants immediately after infestation. Here, we focused on the effects of time after the last infestation on the attractiveness of HIPVs in a tritrophic system consisting of corn plants, *M. separata* larvae, and *C. kariyai*.

Methods and Materials

Plants and Insects Corn plants (*Zea mays* L., Honey Peter 619, Sakata Seed Co., Kanagawa, Japan) were grown separately in small, transparent plastic pots (5.5 cm diam., 7 cm in height) in an incubator (25±1°C, 50–60% relative humidity (RH)) under fluorescent lights (15,000 *lx*, 16 L: 8D photoperiod). Plants were observed daily and watered when needed. In all experiments, 14-d-old corn plants (ca. 30 cm in height) were used.

Both *M. separata* and *C. kariyai* were reared in the Laboratory of Applied Entomology and Zoology, University of Tsukuba, Japan, from cultures obtained from the Center of Ecological Research, Kyoto University in 2001. *Mythimna separata* was reared on an artificial diet (Silkmate® 2(s), Nosan Corporation) under laboratory conditions (25±2°C, 16 L: 8D photoperiod, and 60-70% RH).

The *C. kariyai* population was maintained under the same conditions used for rearing *M. separata*. Individual fourth instars *M. separata* were offered to 2-3-d-old *C. kariyai* females in small Petri dishes (5 cm diam.) for parasitization. Soon after attack, the host larvae were collected and reared with Silkmate® in plastic boxes (10 cm diam., 6 cm in height). These cages were checked daily to provide sufficient artificial diet until egression, which occurred approx. 10 days after parasitoid oviposition into host larvae. A cluster of cocoons of *C. kariyai* was collected and placed in a transparent plastic cage (16×28×17 cm) until emergence of adults, which occurred 5 days after egression from host larvae. Honey droplets on moist cotton wool were provided as food for the parasitic wasps. Mating occurred immediately after emergence. Unless otherwise stated, all experimental parasitoid females were 3-d-old, mated, and well-fed individuals.

Wind Tunnel Bioassay The flight responses of *C. kariyai* to corn plants previously infested with *M. separata* larvae were observed in a wind tunnel (30×30×150 cm; 25–30 cm/s wind velocity; 25±1°C; 60–70% R.H. and 800 *lx* light intensity) (Fukushima et al., 2001). A metal platform (15 cm in height) was placed 50 cm downwind of the test corn plant as a release point for the parasitoid females. Wasps were selected without bias from the cage and individually placed into a Petri dish (4.5 cm diam., 1.5 cm in height) containing small droplets of honey and water 1 h prior to the experiment and then transferred to the bioassay room. The Petri dish was opened, and a parasitoid female was given 5 min to leave the dish, otherwise it was discarded. Females that did not take off within 5 min or did not land on the plant within 5 min from take off were considered as not having been attracted to the test plant. Each female was used once and then discarded. Wind tunnel tests were performed primarily between 10:00 am and 3:00 pm.

We investigated the effect of time after the removal of host larvae from corn plants on the attraction of *C. kariyai* with a wind tunnel. For control, we used uninfested plants. Twenty 4th-instars of *Mythimna separata* were introduced into a plastic cage with a potted corn plant (14-d-old, ca. 30 cm in height). Larvae were allowed to feed until the damaged area reached approximately 30% of the total surface area (feeding period was ca. 30–45 min). Each pot was covered with aluminum foil during the feeding period to prevent feces from falling on the soil. *M. separata* larvae and their associated products then were removed from the plant with a fine camel hair brush. The plant was washed with tap water and used for wind tunnel bioassays.

Flight responses of *C. kariyai* were examined at different time intervals of 0, 3, 6, 24, 48, 72, 96, 120, 144, and 168 h after removal of larvae. After each test, corn plants were placed inside plastic cages (30×30 cm, 50 cm in height) and kept indoors under conditions of 25±1°C; 60-70% R. H. and a 16 L: 8D photoperiod. Plants were watered regularly during the experimental period. For each plant, 5 *C. kariyai* females were tested for each time interval, and the bioassay was replicated 8 times on different days (a total of 40 wasps for each time interval).

Chemical Analyses Headspace volatiles of corn plants were collected at the different times (0, 6, 24, 48, 96, and 144 h) after removal of the host larvae. Six corn plants were grown in a plastic pot (12 cm diam., 10 cm in height) under the same rearing conditions for 14 days. Before volatile collection, corn pots were covered with aluminum foil to eliminate possible volatiles from the pot.

Prior to headspace collection, each plant was placed in a 3 l glass bottle (13 cm diam., 30 cm in height). Plant volatiles were collected on the adsorbent 'HayeSep-Q' (60-80 mesh) (Restek Corporation, USA) for 2 h at a flow rate of 1,000 ml /min. Each treatment was repeated 4 times with fresh

batches of corn plants. The methods used for the collection of volatiles were the same as reported earlier by Takabayashi et al. (1991). Volatile collections were conducted during daytime (from 10:00 to 15:00). After the 2-hr-collection period, traps were rinsed with 200 µl redistilled hexane, and 100 ng of tridecane were added to the extract as an internal standard. After concentrating the extract under a nitrogen stream, aliquots of the extract were injected into a gas chromatograph-mass spectrometer (GC-MS). Identification and quantification of the trapped volatiles were performed using GC-MS (GC; Hewlett Packard 6890) with an HP-5MS capillary column: 0.25 mm i.d., 30 m length, 0.25 µm film thickness, MS: Hewlett Packard 5973 mass selective detector, ion source 70 eV). The GC oven temperature was programmed to increase from 40°C (5 min hold) to 280°C at 15°C/min. The chemical structure of each compound was determined by comparison with the mass spectra and the retention time of authentic chemical samples or by comparison with the Wiley databases (Wiley 7 N and Wiley275) and the database of the National Institute of Advanced Industrial Science and Technology (SDBS compounds and spectral search) (http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi).

Statistics The effect of time after removal of *M. separata* larvae from infested corn plants on the percentage of *C. kariyai* that landed on the plants was analyzed with a mixed model using the restricted maximum likelihood (REML) and individual plants as a random effect. Tukey's HSD test was used to analyze the differences in the percentages of *C. kariyai* that landed on each treatment plant (infested plants) and control plant (intact plants). Proportional data were transformed by arcsine square root before analysis. Linear regression analysis was performed to examine the relationship between the amounts of each compound and the mean percentage of *C. kariyai* females that were attracted to the *M. separata*-infested plants using the least squares method. All analyses were performed with the JMP statistical software (SAS Institute, 2003).

Results and Discussion

Olfactory Responses of Cotesia kariyai to Host-infested Plants The percentage of naive *C. kariyai* females that were attracted to *M. separata*-infested plants (flight response) gradually decreased with an increase in time after the last infestation (Fig. 1). The effects of time after the last infestation were significant (Mixed model; $df=9$, $F=4.462$, $P<0.001$).

When the flight responses were tested within a day (0, 3, 6, 24 h), the percentage of wasps that were attracted to *M. separata*-infested plants was significantly higher than the one attracted to the controls (Fig. 1; Tukey's HSD test, $P=0.05$). Two d after the last infestation and later (48 to 144 h), the flight responses to previously infested plants were not significantly different from those to the uninfested controls or the responses within a day after the last infestation (Fig. 1; Tukey's HSD test, $P=0.05$). When 168 h had passed after the last infestation, the flight response to infested plants was not significantly different from that to the uninfested controls and from responses to plants that experienced the last infestation 48 to 144 h. However, 168 h past infestation, the parasitoids´ response to the previously infested plants was significantly lower than the one observed within a day after the last infestation (Fig. 1; Tukey's HSD test, $P=0.05$). The olfactory responses of *C. kariyai* females to previously infested plants decreased significantly with time only 24 h after termination of infestation; this was the time point of change in attractiveness. Similar results have been reported in a tritrophic system of corn plants, *M. separata* larvae, and the tachnid fly *Exorista japonica* with significantly higher responses of the parasitic fly observed only within 1 day (Hanyu et al., 2009). Kugimiya et al. (2010) showed that *Cotesia vestalis*, a larval parasitoid of diamondback moth (*Plutella xylostella*), preferred Japanese mustard spinach 1 and 2 days after herbivory (formerly infested plants) over intact plants, and freshly infested plants over formerly infested plants.

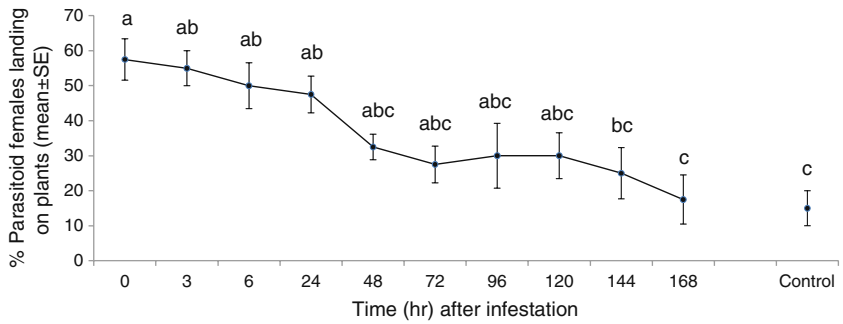


Fig. 1 Wind tunnel bioassay. Percentage of *Cotesia kariyai* females (mean±SE) landing on corn plants previously infested with *Mythimna separata* host larvae. Controls: uninfested corn plants. The same letter indicates no significant difference (Tukey-Kramer HSD test $P=0.05$). $N=40=100\%$ wasps were tested for each time interval

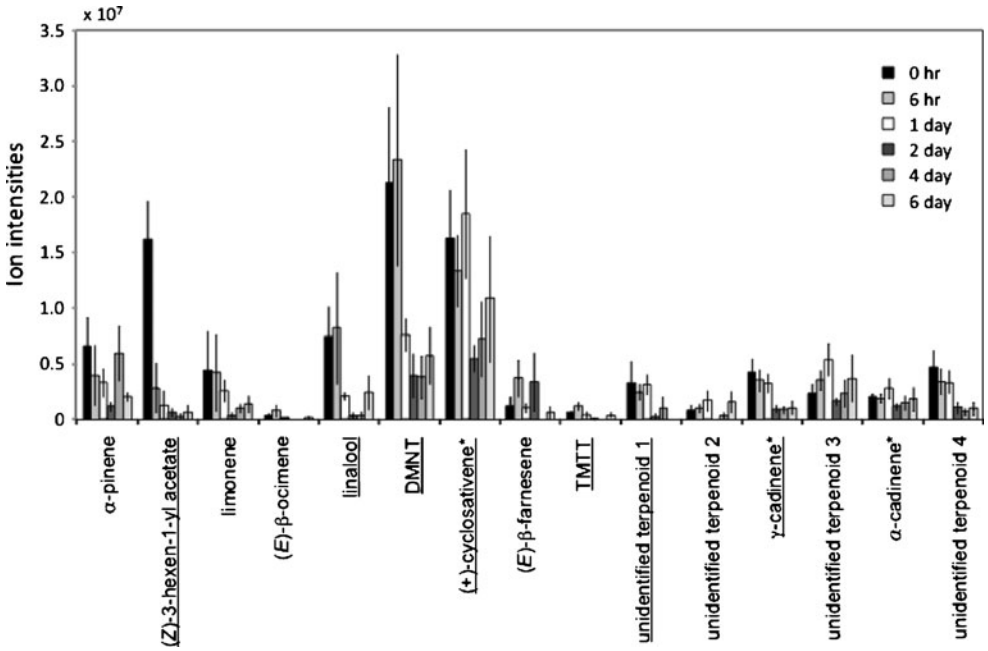


Fig. 2 Volatile compounds identified from the headspace of corn plants previously infested with *Mythimna separata* larvae. Mean±SE ion intensities (N=4). DMNT: (*E*)-4,8-dimethyl-1,3,7-nonatriene. TMTT: (*E, E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Quantities of underlined compounds correlate with attractiveness shown in Fig. 1 (regression analyses). Compounds with * were tentatively identified by comparison with mass spectra from databases

Chemical Analyses In total, 15 compounds were recorded in the headspace of corn plants infested by *M. separata* (Fig. 2). α-Pinene, (*Z*)-3-hexen-1-yl acetate, limonene, (*E*)-β-ocimene, linalool, DMNT ((*E*)-4,8-dimethyl-1,3,7-nonatriene), (*E*)-β-farnesene, (+)-cyclosativene, and TMTT ((*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene) were identified based on the comparisons of the mass spectra and the retention times with authentic samples by GC-MS. γ-Cadinene and α-cadinene were tentatively identified from mass spectra databases. Four compounds remain unidentified. The induced compounds listed in Fig. 2 were not exactly the same as those detected in our previous study (Takabayashi et al., 1995), probably due to a difference in the cultivar (Royal Dent corn in Takabayashi et al., 1995).

The attractiveness of infested corn plants with different times after the last infestation was positively correlated with the total amount of volatiles in the time course (regression analysis, P=0.003). We then tested the correlation of the attractiveness of the plants and the amounts of each volatile compound in the time course. Among 15 compounds, (*Z*)-3-hexen-1-yl acetate, linalool, DMNT, (+)-cyclosativene, TMTT, unidentified compound 1, γ-cadinene, and 4 unidentified compounds showed significant correlations with plant attractiveness (regression analysis) (Table 1). These compounds may cause the differences in attractiveness of plants at different times after infestation. By using this approach, we should be able to select candidates from a herbivore-induced plant volatile blend that are involved in the attraction of natural enemies (Kugimiya et al., 2010).

Previously, we reported that *C. kariyai* females were attracted to (*Z*)-3-hexen-1-yl acetate and α-pinene (Ozawa et al., 2008). The production of α-pinene by the infested plants does not match the change in attractiveness in time (regression analysis; Table 1). The amounts of (*Z*)-3-hexen-1-yl acetate released by the infested plants were significantly different 0 and 24 h after the last infestation, but did not significantly differ 24 and 168 h after stop of infestation (Tukey's HSD test, P<0.001), thus indicating that the change in the production of (*Z*)-3-hexen-1-yl acetate alone does not explain the changes in the attractiveness shown in Fig. 1. Ozawa et al. (2008) reported that *C. kariyai* responded to a mixture of volatiles but that the wasps did not respond to single compounds in the blend. Thus, the

Table 1 Linear regression analysis of the amounts of each compound and the mean percentage of *C. kariyai* females attracted to *M. separata*-infested plants

Compound	r^2 value	t value	P value
α-Pinene	0.0672	1.26	0.2212
(*Z*)-3-Hexenyl acetate	0.4098	3.91	0.0008
Limonene	0.1250	1.77	0.0901
(*E*)-β-Ocimene	0.0974	1.54	0.1376
Linalool	0.2255	2.53	0.0190
DMNT	0.3199	3.22	0.0040
(+)-Cyclosativene	0.1652	2.09	0.0487
(*E*)-β-Farnesene	0.0207	0.68	0.5029
TMTT	0.2680	2.84	0.0096
Unidentified compound 1	0.3255	3.26	0.0036
Unidentified compound 2	0.0091	0.45	0.6569
γ-Cadinene	0.4882	4.58	0.0001
Unidentified compound 3	0.0100	0.47	0.6416
α-Cadinene	0.0474	1.05	0.3070
Unidentified compound 4	0.3277	3.27	0.0035
Total	0.3421	3.38	0.0027

changes in the attractiveness may be explained by changes in the blend of volatiles released by corn plants in the course of time after infestation. Turlings et al. (1990) reported that corn seedlings infested by the beet armyworm *Spodoptera exigua* for 2 h released terpenoids only in minute amounts immediately after infestation, but that larger amounts of the compounds were detected after 16 h (following day). In our study, (*E*)-β-ocimene, (*E*)-β-farnesene, and the 3 unidentified compounds showed a similar trend (Fig. 2).

The host larvae *M. separata* are nocturnal and thus feed on host plants mainly during the night, except for the early instars (Sato et al., 1983). By contrast, *C. kariyai* females search for host larvae during the daytime when the larvae are hiding (Sato et al., 1983; Shiojiri et al., 2006). Under these conditions, the host searching wasps likely use *M. separata*-induced corn plant volatiles induced by the larvae during the previous night. Thus, it would be adaptive for *C. kariyai* to respond not only to volatiles from corn plants freshly damaged by hosts but also to plants with damage caused within a 24-h-period. From the plant's viewpoint, continuing to produce higher amounts of *C. kariyai* attractants for 24 h after the last damage by the host larvae during the previous night also would be adaptive because the plants are likely to be damaged by herbivores in the forthcoming night. However, the metabolic costs of induced volatile production in corn plants is expected to be costly (Hobrallah et al., 2004). Currently undamaged corn plants with a history of previous infestation should stop producing *C. kariyai* attractants at some time. The recorded time period (24 h) after the last infestation during which previously infested corn plants keep on attracting may be counterbalanced by the benefits for the plants and *C. kariyai*. Further, herbivore-induced plant volatiles also may be used by other sympatric herbivore species to search for food plants (Hilker and Meiners, 2006), thus causing additional ecological costs/benefits in the production of herbivore-induced plant volatiles for plants. The ecological relevance of timing of the production of herbivore-induced plant volatiles also has been discussed with respect to egg parasitoids (Hilker and Meiners, 2006). Further studies that clarify the mechanisms involved in timing HIPV production will shed light on the relative costs/benefits of the members of tritrophic interactions.

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Volatiles Mediating a Plant-Herbivore-Natural Enemy Interaction in Resistant and Susceptible Soybean Cultivars

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Abstract Several studies have shown that herbivore-induced plant volatiles act directly on herbivores and indirectly on their natural enemies. However, little is known about the effect of herbivore damage on resistant and susceptible plant cultivars and its effect on their natural enemies. Thus, the aim of this study was to evaluate the attraction of the herbivorous pentatomid bug *Euschistus heros* and its egg parasitoid *Telenomus podisi* to two resistant and one susceptible soybean cultivars with different types of damage (herbivory, herbivory+oviposition, and oviposition). In a Y-tube olfactometer, the parasitoids were attracted to herbivory and herbivory+oviposition damaged soybean plants when compared to undamaged soybean plants for the resistant cultivars, but did not show preference for the susceptible cultivar Silvânia in any of the damage treatments. The plant volatiles emitted by oviposition-damaged plants in the three cultivars did not attract the egg parasitoid. In four-arm-olfactometer bioassays, *E. heros* females did not show preference for odors of damaged or undamaged soybean plants of the three cultivars studied. The Principal Response Curves (PRC) analysis showed consistent variability over time in the chemical profile of volatiles between treatments for the resistant cultivar Dowling. The compounds that most contributed to the divergence between damaged soybean plants compared to undamaged plants were (*E,E*)-α-farnesene, methyl salicylate, (*Z*)-3-hexenyl acetate, and (*E*)-2-octen-1-ol.

Key Words *Euschistus heros* · *Telenomus podisi* · Stink bug · Tritrophic interactions · Indirect defense

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Introduction

Plants are equipped with several mechanisms to defend themselves against herbivores, microbial pathogens, or abiotic stress. One of the mechanisms of damage-induced plant defense is the production of plant volatile organic compounds (VOCs) that serve as repellents to herbivores or as attractants to parasitoids and predators (Price, 1997; De Moraes et al., 2001; Hilker and Meiners, 2006; Chen, 2008; Heil, 2008).

Plants change the blend of VOCs in response to damage either qualitatively or quantitatively (Dicke, 1999), depending mainly on plant traits that vary among species or genotypes and on the different herbivore species causing the damage (Lin et al., 2008; Rasmann and Turlings, 2008). This change can be responsible for differential attraction of parasitoids and predators (Fritzsche Hoballah and Turlings, 2001). Soybean plants, *Glycine max* L. (Fabales: Fabaceae)

and pigeon pea, *Cajanus cajan* (Valder) (Fabales: Fabaceae) damaged by *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae) released similar chemical profiles of volatiles that attracted the egg parasitoid *Telenomus podisi* (Ashmead) (Hymenoptera: Scelionidae). However, when soybean was damaged by feeding *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) larvae, the chemical profile was different and did not attract *T. podisi* (Moraes et al., 2005).

The identification of chemicals responsible for plant defense and their role in the interactions with natural enemies could be used to attract and retain parasitoids in crops (Gatehouse, 2002) and help breeders in the development of cultivars resistant to pests (Piubelli et al., 2005). Therefore, it is important that hostplant resistance interacts positively with the action of natural enemies. However, this association does not always result in positive interactions. Chemical and morphological plant traits can directly influence survival, fecundity, and foraging success of natural enemies on hosts or prey. These traits also can have indirect effects on the quality of herbivores that in turn affects the physiology, behavior, or development of their natural enemies (Cortesero et al., 2000). In a study investigating the effects of chemical plant resistance against herbivores and of predators on plant fitness, Bartlett (2008) observed a negative interaction between the predator *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) and soybean resistance against *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae).

Studies on resistance of soybean cultivars primarily involve direct defense against herbivory during the reproductive stage of soybean (Piubelli et al., 2005; Li et al., 2004, 2008). Carrão-Panizzi and Kitamura (1995) reported that the cultivar IAC100 contains high quantities of isoflavones compared to other soybean cultivars studied and showed high resistance to herbivorous stink bugs. The resistance of IAC100 to stink bugs and lepidopteran larvae was confirmed by McPherson and Buss (2007) and Piubelli et al. (2005) who proposed an antibiotic effect of this cultivar on velvetbean caterpillars, *A. gemmatalis*. The soybean cultivar Dowling shows antixenotic and antibiotic effects against aphids, which stop feeding and cause death after 48 hr (Li et al., 2004, 2008).

However, except for these studies of direct effects of soybean cultivar resistance on herbivores, no study has yet addressed the question how resistant traits of the vegetative stages of soybean cultivars affect the plant's indirect defense against herbivores. For Integrated Pest Management in soybean crops, it has been proposed that natural enemies could be released or attracted to the field before herbivorous stink bugs arrive (Borges et al., 1998). Therefore, it is important that the development of resistant cultivars does not alter plant responses to herbivore damage and the release of herbivory-induced volatiles that attract natural enemies.

In this study, we tested the hypothesis that the level of indirect defense in response to damage by herbivores in resistant soybean cultivars is different from that of susceptible cultivars. Hence, we compared chemically the blend of herbivore-induced volatiles in resistant and susceptible cultivars during the vegetative stages. In addition, we compared the behavioral response of both herbivores and their parasitoids to induced volatiles released by the different cultivars. We chose two different cultivars that are known to display resistance against herbivores (IAC100 and Dowling) and one cultivar that shows high susceptibility to stink bugs (Silvânia) (Laumann et al., 2008). The studied herbivorous insect was the stinkbug *E. heros* and its egg parasitoid *T. podisi*.

Specifically, we studied the following questions: (a) Does *T. podisi* respond differently to resistant and susceptible cultivars when exposed to undamaged plants or plants damaged by herbivory and oviposition by *E. heros*? (b) Are *E. heros* females attracted to the volatiles of undamaged or damaged soybean cultivars? (c) Do resistant and susceptible soybean cultivars release a different chemical profile of volatiles when submitted to herbivory, herbivory+oviposition, or oviposition damage by *E. heros*? (d) Does attraction of *T. podisi* depend on the extent of herbivore damage?

Methods and Materials

Plant and Insect Rearing Soybean (cultivars Dowling, IAC100 and Silvânia) seeds were obtained from the Embrapa Cerrados Research Center (Brasília, DF, Brazil). Seeds were germinated on damp paper, and after 3 day they were transplanted to pots with a mixture of soil and organic substrate (in a proportion of 1:1 *w*/*w*). Plants were kept in a controlled environment room at L14:D10 photoperiod, 27± 1°C and 65±10% r.h. All plants used were in the V3 physiological stage (Fehr et al., 1971).

Euschistus heros individuals were obtained from a laboratory colony started from adults collected in soybean fields near Embrapa Genetic Resources and Biotechnology, Brasília, Brazil (15°47′ S, 47°55′ W). Bugs were reared in 8 l plastic containers, on a diet of soybeans, sunflower seeds (*Helianthus annus*), raw peanuts (*Arachis hypogaea*), fresh green beans (*Phaseolus vulgaris*), and water. The food supply was renewed twice a week. To provide oviposition substrate and shelter for the bugs, a piece of a nylon mesh screen (15 cm^2) was placed inside the cages. To obtain virgin females, insects were sexed after their imaginal molt and cuticular hardening, and maintained isolated from males until their use in experiments.

The egg parasitoid *T. podisi* was obtained from a laboratory colony raised on eggs of *E. heros*. The wasps were maintained in acrylic cages (25 cm^2 tissue culture flask, angled neck; ICN Biomedicals, Irvine, CA, USA), and fed with a drop of honey, supplied twice a week. Once parasitoids hatched from eggs, they were transferred to an egg-free acrylic cage where they could mate for a period of 24 hr. *Euschistus heros* and *T. podisi* were maintained in separate environmental rooms at 27±1°C, 65±10% r.h., and a 14 hr photoperiod.

Preparation of Plants for Bioassays We studied the attraction of *T. podisi* to the three soybean cultivars (cv) that had been subjected to following treatments: (1) herbivory damaged soybean (Herb): five virgin, sexually immature *E. heros* females (2–4 day-old) were placed on the plant; (2) herbivory+oviposition damaged soybean (HerbOvi): five mated *E. heros* females (12–15 day-old) were placed on the plant; (3) oviposition damaged soybean (Ovi): five mated *E. heros* females, with their stylet removed using dissection scissors, were placed on the plant; (4) control: undamaged soybean (UD). For treatments, each plant was kept in a micropore plastic bag (bread bag) where the insects were introduced. Plants were used 24, 48, 72, 96, and 120 hr after damage, with 60 replicates for each time/treatment combination. All insects were removed from the plants a few minutes before the bioassays.

We studied the preference behavior of *E. heros* females by using soybean plants that had been submitted to the same treatments as described above. All bioassays were carried out using plants on the 4th d of damage, because preliminary results showed that natural enemies respond to damaged plants only on the 4th d, and the chemical analysis of the extracts collected from the air-entrainment also showed higher levels of volatiles released on the 4th d

Olfactometer Bioassays with Egg Parasitoids To determine if herbivory- or oviposition-induced volatiles have an effect on the foraging behavior of parasitoids, individual naïve female parasitoids were offered a choice between odors in a Y-shaped olfactometer. A square acrylic block (19×19 cm) with a Y-shaped cavity (1 cm thickness) sandwiched between two glass plates was used as the bioassay arena (Moraes et al., 2005). The trunk of the apparatus measured 8 cm, and each arm 7 cm. Damaged or undamaged plants were placed in glass chambers that were connected to the olfactometer via silicon tubing. Filtered (activated charcoal) and humidified air was pushed through the system at 0.6 l/min, and pulled through at 0.2 l/min, in a push-pull system. The behavior of the insect was monitored by a camera (CCD camera Sony SPT M324CE; Sony, Minato-Ku, Tokyo, Japan fitted with a 12.5–75.0 mm/F1.8 zoom lens) coupled to SACAM software (Jorge et al., 2005) to register behavioral parameters. A single *T. podisi* female was introduced at the base of the Y-tube and observed for 600 sec, and the first choice in each arm was recorded by the software. The first choice was considered to have been made when the insect entered an arm and remained there for at least 20 sec. Each individual was used only once, and the plants were replaced after every five repetitions. The position of the arms of the olfactometer was changed after every five bioassays in order to avoid any bias in the parasitoid response. The response of the parasitoid females was tested for each combination as follows: (1) Herb vs. UD (N=60); (2) HerbOvi vs. UD (N=60), and (3) Ovi vs. UD (N=60). The bioassays were carried out for each soybean cultivar 24, 48, 72, 96, and 120 hr after the start of damage. The damaged and undamaged plants were kept under the same conditions, but in different rooms to avoid plant–plant signalling, until used in the bioassays.

Olfactometer Bioassays with Herbivore Females A four-arm-olfactometer modified from Vet et al. (1983) was used with *E. heros* females. The olfactometer consisted of a square acrylic block (44×44 cm) with an X-shaped cavity (1.5 cm) sandwiched between two glass plates, and it was used as the bioassay arena. The "X" of the apparatus measured 37 cm, and length of the active area for each arm was 25 cm. The length of each arm considered for a positive response of the insects was 15 cm, measured from the upwind end towards the center of the olfactometer. The bioassay procedure was the same as described for the Y-olfactometer above. The first choice was considered to have been made when the insect entered an arm and remained there for at least 60 sec. The responses of 70 stink bug females were tested for each combination: (1) Herb vs. HerbOvi vs. Ovi vs. UD for each soybean cultivar (cv) with 96 hr of damage and (2) UD of cv Dowling vs. UD cv IAC100 vs. UD cv Silvânia vs. air. For each combination, we tested females in different physiological conditions: (a) starved virgin females (the diet was removed 24 hr before the bioassay); (b) fed virgin females; and (c) fed mated females.

Volatile Analysis Herb-, HerbOvi-, Ovi-, and UD-plants were placed individually in cylindrical glass chambers (internal volume 10 l). Volatiles were collected for 24 hr, during seven consecutive days (N=11 replicates for each treatment). In an attempt to minimize contamination by volatiles from the soil, pots were wrapped in aluminium foil. A glass tube containing the adsorbent Super Q (100 mg, 80-100mesh, Alltech PA, USA) was connected via a PTFE tube to a vacuum pump at a flow of 0.6 l/min, and the air entrance was connected to a flow of air filtered with activated charcoal (1.0 l/min) creating a positive push-

pull system (Moraes et al., 2008). The trapped volatiles were eluted from the adsorbent using 500 µl *n*-hexane and pre-concentrated to 200 µl under a N_2 flow. Extracts were stored at −20°C until analysis by gas chromatography (GC) and GC coupled to mass-spectrometry (GC-MS).

The extracts obtained by air entrainment were analyzed by GC (Shimadzu 17A, DB-5 column, 30 m×0.25 mm ID, 0.25 µm film, J&W Scientific, Folsom, CA, USA), using a temperature program of 50°C/1 min, 15°C/min to 250°C/20 min. The column effluent was analyzed with a flame ionization detector (FID) at 270°C. For the GC analyses, 50 µl of each extract were separated, and 1 µl of 1-octen-3-ol was added as internal standard (IS), for a final concentration of 9.8 µg/ml of the IS. One microliter of each sample was injected in splitless mode with helium as carrier gas. Amounts released by the plant in each 24 hr period were calculated in relation to the area of the internal standard. Data were collected with ClassGC software and were handled using Excel Software (Microsoft Corporation 2003).

For qualitative analysis, selected extracts were analyzed using a Shimadzu QP2010 instrument equipped with a quadrupole analyzer, a non-polar DB-5 column (30 m× 0.25 mm ID, 0.25 µm film, J&W Scientific, Folsom, CA, USA) and a splitless injector, with helium as the carrier gas. Ionization was by electron impact (70-eV, source temperature 200°C). Data were collected and analyzed with GC-MS Solutions 2.4 Software (Shimadzu Corporation, Japan). Identifications were made by comparison of spectra with library databases (NIST 2005) or with published spectra and confirmed by using authentic standards when available.

Chemicals Super Q (80/100 mesh) was purchased from Alltech (PA, USA). n-Hexane (95% pesticide residue) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). (*E*)-2-Hexenal and (*Z*)-3-hexen-1-ol were purchased from Sigma Aldrich (Gillinghan, Dorset, UK). (*Z*)-3-Hexenyl acetate was purchased from Alfa Aesar (Heysham, UK) and (*E*)-ocimene from Botanix (Paddock Wood, Kent, UK). Limonene and linalool were purchased from TCI America (Portland, OR, USA). Methyl salicylate, β-caryophyllene, benzothiazole, β-myrcene, geranyl acetone, and (*E*)-2-hexenyl acetate were purchased from Sigma Aldrich (Steinheim, Germany). (*E*,*E*)-α-Farnesene was provided by Jeffrey Aldrich (USDA-ARS, USA) and (*E*)-4,8-dimethylnona-1,3,7-triene (DMNT) and (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) were provided by Michael Birkett (Rothamsted Research, UK).

Statistical Analysis The null hypothesis of equal preference (P=0.05) of parasitoids for the volatiles from damaged plants vs. undamaged plants was tested at each sampling time (24, 48, 72, 96, and 120 hr), using *chi-square* tests with the Yates correction for continuity (Zar, 1998).

Since the volatile sampling was carried out on the same plant at several time points, repeated measures analysis of variance was used to avoid the problem of pseudo-replication during the times analyzed (Hurlbert, 1984; Green, 1993). Therefore, the total amounts of volatiles released in each treatment over time were compared by MANOVA (PROC ANOVA with the PROFILE statement; SAS Institute, 2001), using the subsequent sampling times as repeated measures of the same experimental unit, as suggested by von Ende (1993). Significant data by MANOVA were subjected to *post-hoc* mean comparisons in individual sampling time using the Tukey's test (significance level 5%).

The change in the chemical profile of damaged and undamaged soybean plants over time was assessed using Principal Response Curves (PRC) analysis. PRC models the effect of the treatment d at a determined time t in a species k. The statistical model for compound concentration is: $(y_{d(j)tk}=y_{\underline{0}k}+b_k\ c_{dt}+\varepsilon_{d(j)tk})$, where, $y_{d(j)tk}$ is the concentration of the compound k in replicate j of treatment d at time t, $y_{\underline{0}tk}$ is the mean concentration of the compound k in time t in the control (untreated plant, d = 0), c_{dt} is a basic response pattern for every treatment d, and time t, b_k is the weight of each compound with this basic response pattern, and $\varepsilon_{d(j)tk}$ is an error term with mean zero and variance σ_k^2. These parameters were generated through redundancy analysis (RDA) least-squares estimates from reduced-rank regression with concomitant variables (van den Brink and ter Braak, 1999), with N=11 replicates for each sampling time and treatment.

The values of c_{dt} for the treatment (treatment effect) were plotted as vertical axis (first principal component, PRC1) over sampling time generating a PRC diagram, with a curve for each treatment, that describes the dynamic changes in the blend of volatiles released by the plant. We compared the variation in volatile composition in damaged and undamaged soybean, the latter being our standard (c_{dt} = 0). The response variable weights (displayed on the right side of each diagram) allow evaluation of the contribution of each compound to the overall blend composition. The higher the values of the weight of the variable, the higher the contribution of the compound to the actual response pattern of the PRC.

PRC analysis was applied separately to each soybean cultivar, comparing the damaged and undamaged soybean. For each set of analyses, the significance of each PRC diagram was tested using an F-type test obtained by permutating (Monte Carlo permutation tests; 999 permutations) whole time series in the partial RDA from which the PRC was obtained. A second series of permutation tests (individual contrasts) was performed within each sampling date to determine if differences indicated by the PRC diagram between treatments were statistically significant.

These analyses were carried out using the software CANOCO 4.5 for Windows (ter Braak and Šmilauer, 2002). The data from the main volatile compounds responsible for the treatment differences in PRC diagram were individually submitted to repeated measures MANOVA followed by contrasts formed within sampling time to compare induction treatments against the control, using the *t* test (significance level 5%).

Results

Olfactometer Bioassays with Egg Parasitoids When testing the resistant soybean cultivar Dowling, the egg parasitoid *T. podisi* responded preferentially to the odor of plants with herbivory (Herb; χ^2=7.67, *P*=0.005; χ^2=8.06, *P*=0.005) and herbivory plus egg deposition (HerbOvi; χ^2=9.60, *P*=0.002; χ^2=8.06, *P*=0.005) when compared to undamaged plants (UD) after a 72 and 96 hr treatment-period, respectively (Fig. 1). *Telenomus podisi* preferred volatiles from the resistant soybean cultivar IAC100 when these have been treated for 96 hr by herbivory (Herb, χ^2=11.26, *P*=0.001) and herbivory plus egg deposition (HerbOvi, χ^2=8.06, *P*=0.005) to volatiles from undamaged (UD) plants. No preference was observed to the odors of cv IAC100 with other treatments at the different times evaluated (Fig. 2). For the susceptible cultivar Silvânia, *T. podisi* did not discriminate between odors emitted by undamaged plants and damaged plants regardless of the treatments and duration of treatment (*P*>0.05) (Fig. 3).

Olfactometer Bioassays with Herbivore Females In the four-arm-olfactometer bioassays, *E. heros* females did not show a preference for the Herb-, HerbOvi-, Ovi-, or UD-plants (χ^2, *P*>0.05) of any the three cultivars Silvânia, IAC100 and Dowling, or for undamaged plants of the three cultivars tested against air. This result was independent of the physiological state of the females (virgin starved, virgin fed, and mated fed).

Volatile Analysis The total amount of volatiles released by the soybean cultivar Dowling differed between treatments throughout the sampling times (treatment: *F*=9.89; *d.f.*=3, 56; *P*=0.001; interaction: *Wilk's* λ=0.383; *d.f.*=18, 145; *F*=3.25; *P*=0.001) (Fig. 4). The chemical profile of Dowling plants treated by herbivory (Herb), herbivory plus egg deposition (HerbOvi), egg deposition only (Ovi) differed when compared to undamaged plants (UD) at 48 hr of treatment and at longer treatment periods (*post-hoc* pairwise comparisons by Tukey test, *P*<0.05). No difference was observed for treatment effects or its interaction with time for the cv IAC100 (treatment: *F*=1.82; *d.f.*=3, 56; *P*=0.15; interaction: *Wilk's* λ=0.583; *d.f.*=18, 145; *F*=1.61; *P*=0.06) or cv Silvânia (treatment: *F*=2.63; *d.f.*=3, 56; *P*=0.06; interaction: *Wilk's* λ=0.607; *d.f.*=18, 145; *F*=1.55; *P*=0.08) (Fig. 4).

The chemical profile of the extracts obtained from the treatments Herb, HerbOvi, Ovi, and UD differed between the three cultivars Dowling, IAC100, and Silvânia. The compounds (*Z*)-2-octen-1-ol and (*E*)-2-octen-1-ol were identified only in cv Dowling; (*E*)-2-hexenyl acetate, and indole were identified in cv IAC100, while DMNT was

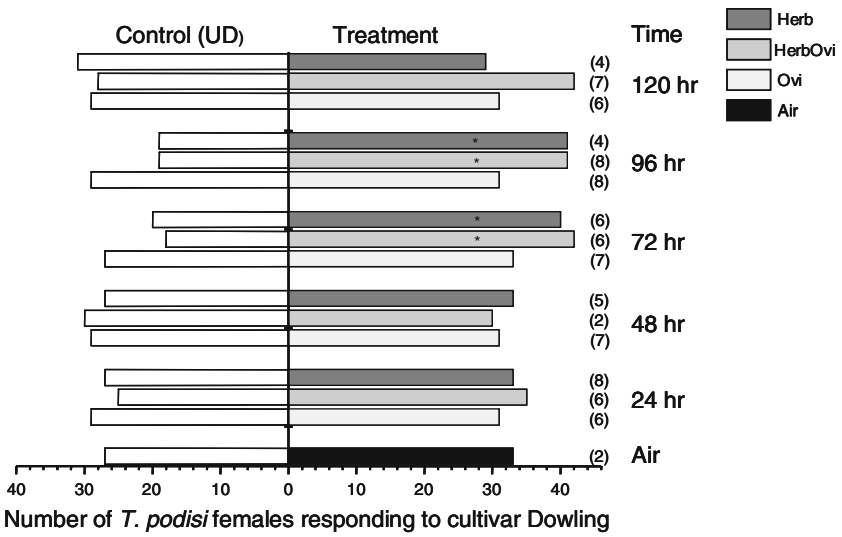


Fig. 1 Responses of the egg parasitoid *Telenomus podisi* in a Y-tube olfactometer to volatiles from soybean cultivar Dowling subjected to different treatments. *UD* undamaged soybean, *Ovi* soybean plant with oviposition damage, *HerbOvi* soybean plant with herbivory +oviposition damage, *Herb* soybean plant with herbivory damage. 24, 48, 72, 96 and 120 hr: time of plant treatment (induction time). *Asterisks* indicate significant differences (*P*<0.05) between parasitoid responses to volatiles from different treatments: χ^2 test on the proportion responding. *Numbers in brackets* represent the number of insects that did not respond to either of the treatments tested

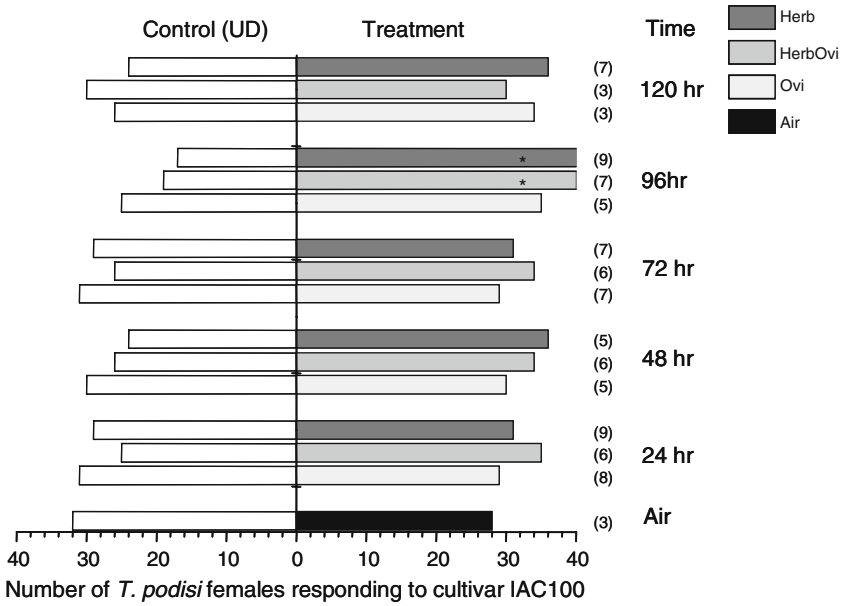


Fig. 2 Responses of the egg parasitoid *Telenomus podisi* in a Y-tube olfactometer to volatiles from soybean cultivar IAC100 subjected to different treatments. *UD* undamaged soybean, *Ovi* soybean plant with oviposition damage, *HerbOvi* soybean plant with herbivory+oviposition damage, *Herb* soybean plant with herbivory damage. 24, 48, 72, 96 and 120 hr: time of plant treatment (induction time). *Asterisks* indicate significant differences ($P<0.05$) between parasitoid responses to volatiles from different treatments: χ^2 test on the proportion responding. *Numbers in brackets* represent the number of insects that did not respond to either of the treatments tested

identified only in cv Silvânia (Table 1). The cultivars also emitted common compounds, such as the green leaf volatile (*Z*)-3-hexenyl acetate; the terpenoids camphene, limonene, α-humulene, β-caryophyllene, (*E,E*)-α-farnesene, TMTT, and the shikimic acid derivatives benzothiazole, benzaldehyde, and methyl salicylate. In addition, some compounds were released in higher quantities by damaged soybean when compared to undamaged soybean (Table 1).

Soybean Cultivar Dowling The PRC analysis of the plant volatiles released showed that cv Dowling had a consistent variability over time between treatments. Of the total variance in the blend composition of volatiles released, 8.6% is explained by sampling times and 12% is explained by induction treatments. The variance exhibited in the first PRC axis was highly significant (Monte Carlo permutation test $F=35.0$, $P=0.001$), and explained 63.0% of the variation in the blend composition due to interactions between sampling times and induction treatments (Fig. 5). At 24 hr after damage, the analysis did not show a significant difference, indicating that the blends of the four treatments were similar. From 48 hr onward, PRC showed a

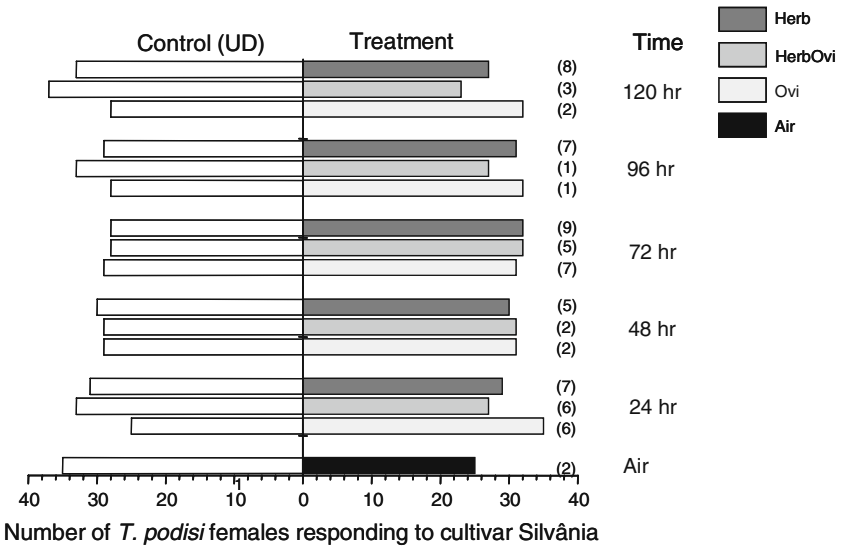


Fig. 3 Responses of the egg parasitoid *Telenomus podisi* in a Y-tube olfactometer to volatiles from soybean cultivar Silvânia subjected to different treatments. *UD* undamaged soybean, *Ovi* soybean plant with oviposition damage, *HerbOvi* soybean plant with herbivory +oviposition damage, *Herb* soybean plant with herbivory damage. 24, 48, 72, 96 and 120 hr: time of plant treatment (induction time). *Asterisks* indicate significant differences ($P<0.05$) between parasitoid responses to volatiles from different treatments: χ^2 test on the proportion responding. *Numbers in brackets* represent the number of insects that did not respond to either of the treatments tested

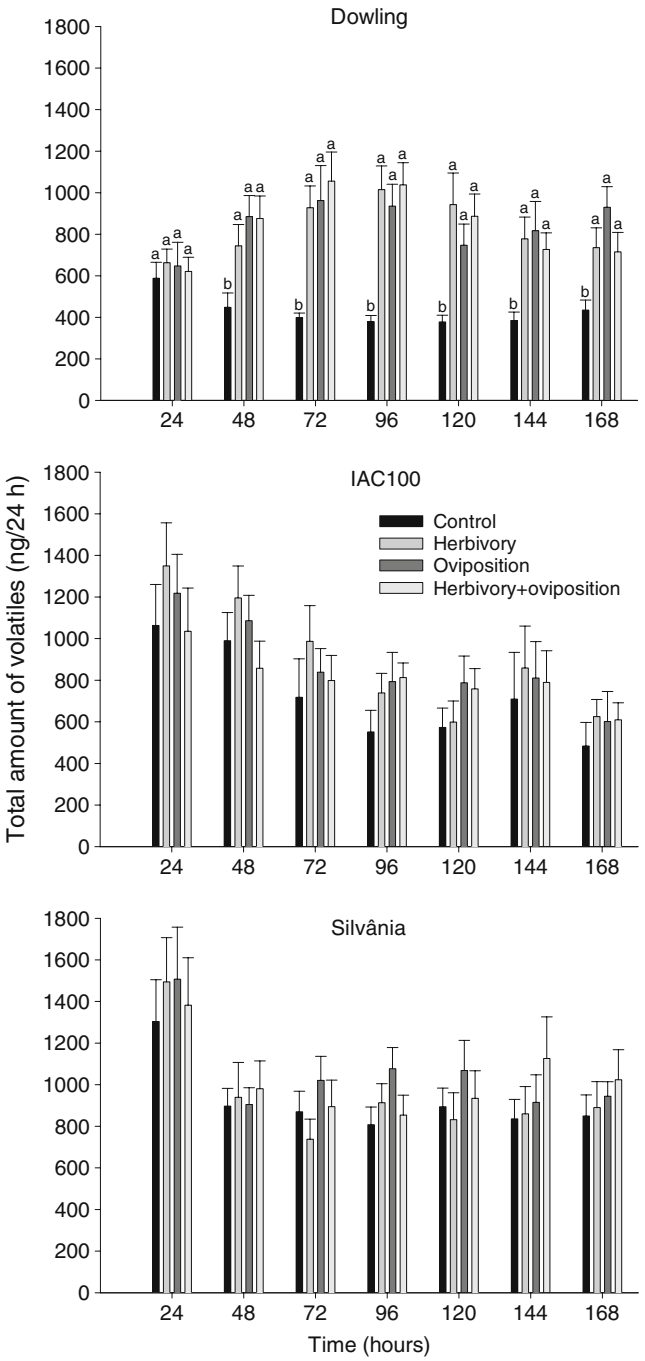


Fig. 4 Total amount of volatiles (mean + SEM) obtained from undamaged plants (control) and plants submitted to the treatments herbivory, oviposition, and herbivory+oviposition for soybean cultivars Dowling, IAC100, and Silvânia. The increase in the amount of volatiles released by damaged plants of cultivar Dowling was significant after 48 hr. For cultivars IAC100 and Silvânia there was no significant difference between treatments and its interactions with time (MANOVA for repeated measures, $P>0.05$). Means followed by the same letter within each sampling date are not significantly different by the Tukey's test at $P>0.05$

clear divergence in curves of the treatments when compared to the undamaged soybean (Fig. 5).

All variable weights (b_k) showed on the right side of the PRC diagram were positive (Fig. 5), which means that all compounds were positively associated with the pattern of changes in the volatile blend. Compounds with weight values higher than 0.5 contribute to the overall volatile blend response (van den Brink and ter Braak, 1999) and the greater the b_k value, the higher the influence on the blend response. Thus, (*E*,*E*)-α-farnesene, methyl salicylate, (*Z*)-3-hexenyl acetate, and (*E*)-2-octen-1-ol were the main compounds that contributed (b_k absolute values >1.0) to the divergence between treatments and control for the cv Dowling (Fig. 5).

A significant interaction between treatment and time (MANOVA for repeated measures, $P<0.05$) was found when data for the main compounds of cultivar Dowling were analyzed individually (Fig. 6). Plants with the treatments Herb and HerbOvi differed from UD plants (contrasts within sampling date; *t* test, $P<0.05$), releasing significantly higher quantities of the same compounds as indicated by PRC analysis (Fig. 6).

Soybean Cultivars IAC100 and Silvânia No significant treatment effect on the volatile blend composition was observed in PRC analysis for the cultivars IAC100 ($F=1.9$, $P=0.073$; Fig. 7, Table 1) and Silvânia ($F=10.4$, $P=0.104$; Fig. 8, Table 1). The variable weights (b_k) displayed in the PRC diagrams showed low values, ranging between −0.5 and 0.5, for all compounds sampled (Figs. 7 and 8).

Although no differences were observed by PRC for IAC100, some compounds were released in higher quantities by plants with the treatments Herb and HerbOvi when compared to UD and Ovi (Table 1). When we analyzed the compounds individually (MANOVA for repeated measures, $P<0.05$), (*Z*)-3-hexenyl acetate ($t_{59,3}=2.031$. $P=0.055$), methyl salicylate ($t_{59,3}=2.159$, $P=0.035$), and (*E*,*E*)-α-farnesene ($t_{48,3}=1.909$, $P=0.061$) were released in significantly or marginally non-significantly higher amounts from plants subjected to 72-hr herbivory (Herb) when compared to undamaged plants (UD). (*Z*)-3-Hexenyl acetate ($t_{59,3}=2.071$, $P=0.043$), methyl salicylate ($t_{59,3}=1.834$, $P=0.072$) and (*E*,*E*) α-farnesene ($t_{55,3}=2.820$, $P=0.006$) were released in significantly or marginally non significantly higher amounts from plants subjected to 96 hr herbivory-plus-oviposition (HerbOvi) when compared to undamaged ones (UD). (*E*,*E*)-α-farnesene ($t_{55,3}=2.322$, $P=0.023$) was released in significantly higher amounts from plants after 96 hr herbivory (Herb) when compared to undamaged plants (UD). The quantities of compounds released by Silvânia also were analyzed by MANOVA for repeated measures, but they did not differ among treatments.

Table 1 Volatiles from differently treated soybean cultivars Dowling, IAC100, and Silvânia

Compounds	Amounts of volatiles [ng 72–96 hr][a]			
	Treatment[b] UD	Herb	Ovi	HerbOvip
Dowling				
Camphene	14.8±2.4	17.9±3.8	37.1±9.6	26.4±8.2
Benzaldehyde	13.6±2.9	20.5±4.2	18.4±5.1	27.4±9.9
(*Z*)-3-Hexenyl acetate	92.5±19.0a	226.4±64.9b	185.0±39.2a	278.6±51.4b
Limonene	31.2±6.8	41.1±8.0	62.9±11.9	48.0±9.5
(*Z*)-2-Octen-1-ol[c]	20.2±2.0a	39.4±10.9a	85.4±16.9b	70.7±17.2b
(*E*)-2-Octen-1-ol[c]	50.6±8.5a	117.3±21.4b	218.9±52.9b	204.8±60.6b
Methyl salicylate	115.3±8.4a	437.8±43.8b	240.8±39.5b	462.6±61.4b
Benzothiazole	13.4±2.4	24.6±3.6	35.7±14.1	15.0±2.6
(*E*)-2-Decenal	9.1±3.4	5.5±1.3	17.9±4.4	16.7±4.8
β-Caryophyllene	30.2±8.5	54.2±13.2	57.8±12.9	50.6±11.5
α-Humulene	42.6±7.0	52.0±10.6	92.1±22.0	85.6±16.9
(*E*,*E*)-α-Farnesene	213.8±24.0a	623.4±75.1b	526.2±74.8b	562.2±66.9b
TMTT	55.9±10.7	114.0±36.8	94.2±25.9	90.9±22.9
IAC100				
(*E*)-2-Hexenal	16.6±7.9	32.0±18.6	12.2±6.3	39.1±18.1
(*E*)-2-Hexen-1-ol	24.7±10.4	12.2±4.4	15.1±6.4	13.5±4.4
(*Z*)-3-Hexen-1-ol	29.8±18.8	10.6±2.6	20.3±12.5	10.7±2.4
Camphene	36.7±13.4	48.4±22.2	47.6±16.9	29.1±11.5
Benzaldehyde	37.2±18.1	19.1±4.7	32.1±12.8	19.9±4.2
(*Z*)-3-Hexenyl acetate	40.8±16.3a	123.3±52.6b	73.3±19.0a	97.2±30.9b
(*E*)-2-Hexenyl acetate	22.7±16.8	52.8±34.9	26.4±11.9	17.2±1.7
Limonene	87.7±37.9	54.5±13.3	110.6±31.8	64.4±22.5
(*E*)-Ocimene	51.1±13.3	65.8±15.6	102.3±60.4	112.5±18.3
Linalool	64.5±18.4	94.0±22.9	166.3±58.8	118.9±22.7
Methyl salicylate	36.6±15.2a	123.9±9.8b	78.9±30.8a	135.1±50.1b
Benzothiazole	15.7±4.7	21.3±42.6	14.5±4.3	16.7±4.2
Indole	20.0±8.2	12.6±3.7	21.0±8.2	19.7±4.2
β-Caryophyllene	101.9±43.5	89.2±5.0	83.6±21.3	90.3±22.3
Geranyl acetone	107.7±36.5	91.3±28.3	122.5±27.2	105.8±26.8
α-Humulene	271.9±68.7	126.9±45.3	198.4±65.4	279.2±77.2
(*E*,*E*)-α-Farnesene	149.5±42.7a	362.9±86.8b	247.9±64.3a	354.2±62.4b
TMTT	67.3±24.1	82.9±18.9	84.1±20.3	70.3±17.9
Silvânia				
(*E*)-2-Hexenal	26.1±12.1	52.7±19.8	64.7±23.6	47.6±18.7
(*E*)-2-Hexen-1-ol	35.7±7.6	20.9±6.0	41.2±11.4	45.5±13.1
(*Z*)-3-Hexen-1-ol	26.2±7.1	20.8±9.8	42.4±12.8	32.8±10.2
Camphene	42.9±6.1	83.2±36.2	79.6±24.9	46.5±7.7
Benzaldehyde	21.0±4.3	19.0±2.7	50.2±11.3	48.0±14.2
(*Z*)-3-Hexenyl acetate	86.2±19.4	52.1±22.9	75.6±24.5	76.4±30.9
Limonene	74.4±16.9	96.9±18.6	175.3±38.8	137.2±28.6
(*E*)-Ocimene	105.5±22.2	119.7±26.1	179.9±18.9	152.9±32.9
Linalool	134.7 ±20.6	144.9±24.0	169.3±27.9	141.5±21.3
DMNT	41.5±11.7	46.2±11.1	61.0±9.9	44.0±9.2
Methyl salicylate	30.1±8.8	46.3±11.8	45.9±11.3	46.5±11.6
Benzothiazole	13.9±2.9	19.4±6.3	13.4±3.5	23.7±8.9
β-Caryophyllene	31.3±9.2	92.1±37.2	41.5±13.1	42.7±18.2

Table 1 (continued)

Compounds	Amounts of volatiles [ng 72–96hr][a]			
	Treatment[b] UD	Herb	Ovi	HerbOvip
Geranyl acetone	138.8±30.8	164.5±29.2	167.1±38.5	138.6±38.3
α-Humulene	140.0±27.3	192.6±39.8	226.0±62.9	219.7±53.0
(*E*,*E*)-α-Farnesene	153.8±25.1	123.3±19.2	222.8±46.5	201.8±44.4
TMTT	66.7±15.8	44.6±15.1	72.4±19.4	49.8±17.4

[a] Mean values (± SEM) calculated from 22 samples obtained by aerations during the period spanning 72-96 hr after the start of damage

[b] (UD) undamaged plants; or plants submitted to treatments Herbivory (Herb), Oviposition (Ovi) and Herbivory+Oviposition (HerbOvi) damage. Means followed by the same letter within a line are not significantly different by the MANOVA at $P>0.05$. TMTT ((*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene), DMNT ((*E*)-4,8-dimethylnona-1,3,7-triene)

[c] Tentatively identified compounds

Discussion

In this study, by comparing the susceptible cultivar Silvânia and the resistant cultivars Dowling and IAC100, it is apparent that each cultivar has a different response to herbivore damage, eliciting different responses by the egg parasitoid. The results support our hypothesis that the level of indirect defense in response to damage by herbivores in resistant soybean cultivars is different from that of susceptible cultivars. We could show that the resistant cultivars (here Dowling and IAC100) showed stronger induction of indirect defense; they show a higher production of volatile compounds in response to herbivore attack and a stronger response by the egg parasitoid, *T. podisi*, to volatiles released from plants with herbivory and herbivory+oviposition when compared to undamaged plants. In addition, the results obtained in this study agree with those presented by previous work on induced plant responses to oviposition by Pentatomidae (Colazza et al., 2004a, b; Moraes et al., 2008), where oviposition itself (without herbivory) did not induce changes of plant odors that are attractive to the egg parasitoids.

However, egg-laying by the pentatomid *E. heros* on the cultivars Dowling and IAC100 changes the plant volatile profile; we have detected differences in the

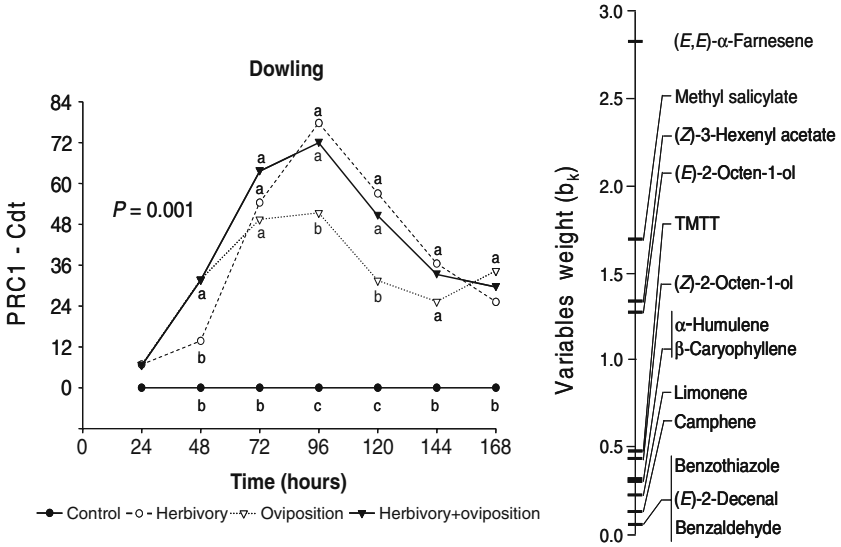


Fig. 5 PRC diagram and variables weights (b_k) based on volatile blends released by soybean plants of the cultivar Dowling, on seven sampling days. The lines represent the response pattern of soybean plants to different treatments in time. The *P*-value indicates significance of the PRC diagram over all sampling times based on *F*-type permutation test. On each date, values of C_{dt} differ significantly between treatments that do not share the same letter code (*a*–*c*); shared or omitted letter codes denote contrasts that do not differ significantly by the Monte Carlo permutation tests at $P<0.05$. The higher (absolute values) the variable weight, the more closely the compound response pattern follows the deviation pattern (from the control, $C_{dt} = 0$ line) indicated on the PRC

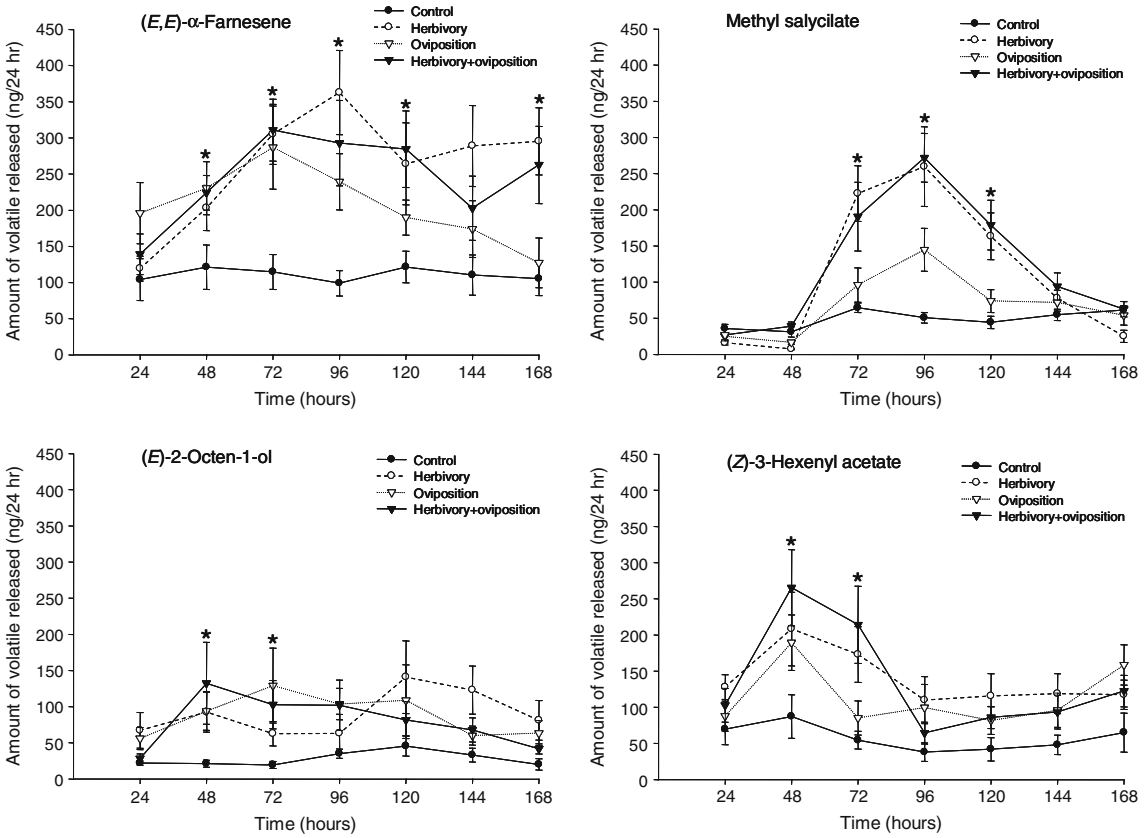


Fig. 6 Amounts (mean ± SEM) of principal volatile compounds released by undamaged soybean (control) and soybean plants submitted to herbivory, oviposition and herbivory+oviposition damage, for cultivar Dowling. *Bars* represent the standard error of the mean. Means marked with an *asterisk* indicate significant differences ($P<0.05$) between induction treatments and control (within a sampling date), *t* test

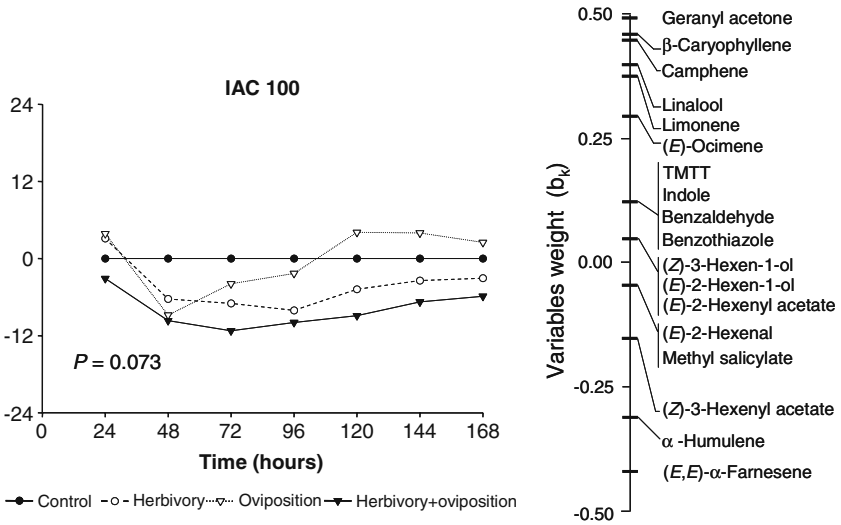


Fig. 7 PRC diagram and variables weights (b_k) based on volatile blends released by soybean plants of the cultivar IAC 100, on seven sampling occasions. The *lines* represent the response pattern of soybean plants to different treatments in time. The *P*-value indicates significance of the PRC diagram over all sampling times based on *F*-type permutation test. The first PRC did not display a significant part of the treatment variance (Monte Carlo permutation test, $P>0.05$), indicating that no significant induction treatment effect on the volatile blend composition was detected. All compounds showed low values for the variables weights ($b_k<0.5$)

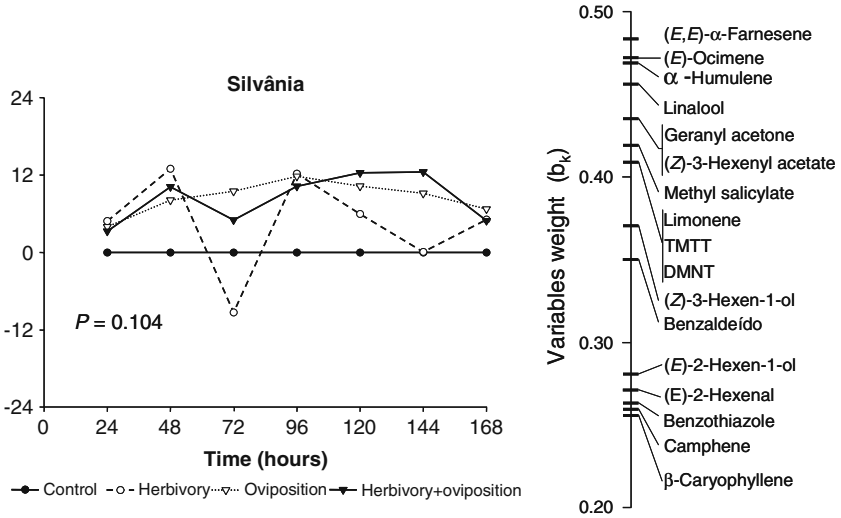


Fig. 8 PRC diagram and variables weights (b_k) based on volatile blends released by soybean plants of the cultivar Silvânia, on seven sampling occasions. The lines represent the response pattern of soybean plants to different treatments in time. The *P*-value indicates significance of the PRC diagram over all sampling times based on *F*-type permutation test. The first PRC did not display a significant part of the treatment variance (Monte Carlo permutation test, $P>0.05$), indicating that no significant induction treatment effect on the volatile blend composition was detected. All compounds showed low values for the variables weights ($b_k<0.5$)

volatile blends of these cultivars between undamaged, herbivory- and herbivory+oviposition-damaged soybean plants. Moraes et al. (2008) suggested that the egg parasitoid *T. podisi* was not attracted to the blend of volatiles produced by herbivory+oviposition and oviposition damaged soybean (cv BR-16), probably due the switching-off of jasmonate signaling caused by oviposition damage. In contrast, the results presented here suggest that oviposition did not switch off jasmonic acid-dependent plant defense because herbivory+oviposition-damaged soybean plants also attracted the natural enemy.

The lack of attraction of the egg parasitoid to oviposition-damaged plants could be explained if the response of the plant is not systemic. When the stink bug lays eggs on the leaves, there is a localized necrosis below and around the eggs (personal observation), which suggests a hypersensitive response (HR) by the plant, isolating the damaged area in a way similar to plant-pathogen responses (Walling, 2000; Kaloshian and Walling, 2005). What induces soybean plants to respond with necrosis to oviposition needs further study, but could be due to microorganisms or chemicals from the oviposition secretion that induce the HR. We are trying to elucidate this phenomenon by studying the chemistry of the saliva and the oviposition secretions.

The PRC analysis used in this work showed the main compounds induced by herbivore damage and the interaction between the damage time and the volatiles released by the soybean plant. The PRC analysis is a multivariate method that is commonly used to aid in impact assessments of pollutant stress on aquatic and terrestrial biological communities in ecotoxicological studies (van den Brink and ter Braak, 1999; van den Brink et al., 2003), and application to other ecological aspects (Torres and Ruberson, 2005; Poelman et al., 2010) has proceeded rapidly. The great advantage of the PRC compared to other multivariate analysis is that it is possible to evaluate the variation of the compounds over time and the importance of each compound in a blend. Furthermore, there is no limitation on the number of variables and normality of the data is unnecessary.

The PRC results suggest that the increase of (*E*,*E*)-α-farnesene, methyl salicylate, (*Z*)-3-hexenyl acetate, and (*E*)-2-octen-1-ol in the total blend have an effect on parasitoid behavior, but the role of these compounds, individually, on the attraction of the egg parasitoid *T. podisi* needs to be evaluated. The attraction of parasitoids and predators to herbivore induced volatiles is often attributed to the relative contribution of each compound in the blend (D'Alessandro et al., 2006, 2009; Pareja et al., 2009), and not all herbivore-induced compounds are important for changing insect behavior. For example, two important compounds in the herbivore-induced volatile blend, indole in maize, *Zea mays*, and methyl salicylate in *Arabidopsis* sp, were not the key compounds responsible for the attraction of the parasitoids tested (D'Alessandro et al., 2006, 2009; Snoeren et al., 2010).

Telenomus podisi did not distinguish among the odors of damaged and undamaged plants for any treatment in cv Silvânia, probably because the volatile compounds released by undamaged and damaged Silvânia soybean are too similar for a discrimination. Vet et al. (1998) suggested that

an insect does not discriminate between odor sources because it is not able to do so, or because the odor is not in the right conditions for the discrimination, i.e., the compounds released by the plant are not in the correct concentration or proportion to be perceived by the natural enemies as a cue indicating the presence of the host. The egg parasitoid *T. podisi* discriminated between volatiles released from differently treated cv Dowling and IAC100, respectively. However, no preference for odor of any treatment of the susceptible Silvânia cultivar was detected; thus, we suggest that the susceptible cultivar was not induced by herbivore damage.

Herbivory-induced plant volatiles are important cues for host foraging behavior of parasitoids. Therefore, maintaining and even enhancing the attractiveness of crop plants to natural enemies should be considered an important trait when breeding new crop varieties. However, these possibilities may sometimes be constrained by the effect of these changes on the attractiveness of the plant to herbivorous pests, as volatiles that attract more natural enemies could also attract more herbivores (Cortesero et al., 2000). Thaler (1999) mentioned that it is advantageous for herbivores to avoid laying eggs on induced plants because such plants are likely to host other herbivores, which are potential competitors for second offspring herbivores, and can also attract a population of natural enemies. The results obtained in this work showed that *E. heros* does not use herbivore-induced volatiles as cues to distinguish between damaged and undamaged plants, or to distinguish between cultivars, but *E. heros* females responded preferentially to undamaged soybean (χ^2, P=0.002) compared to air, suggesting that *E. heros* uses the volatiles from soybean plants to locate its host-plant (results not shown).

The cv Dowling and IAC100 are known to have in their reproductive stage an antixenotic and antibiotic effect against aphids and stink bugs, respectively (Carrão-Panizzi and Kitamura, 1995; Li et al., 2004, 2008). Recently, field experiments showed that population levels of stink bugs colonizing cv Dowling and cv IAC100 were lower than on cv Silvânia suggesting a differential resistance/susceptibility (Laumann et al., 2008). Different studies suggested that the resistance during the reproductive stage of soybean could be related to the amount of isoflavonoids (Rao et al., 1990; Hoffmann-Campo et al., 2001; Piubelli et al., 2003, 2005). Experiments are needed to determine the amount of isoflavonoids during the different physiological stages of soybean plants to evaluate if there is a relation between direct and indirect defense and the quantities of volatile organic compounds.

In summary, the results suggest that the herbivorous stink bug *E. heros* induces the indirect defense of the resistant soybean cultivars Dowling and IAC100, and that the egg parasitoid *T. podisi* is attracted to the volatiles released by herbivory- and herbivory+oviposition-damaged plants of these cultivars. In olfactometer bioassays, *E. heros* did not distinguish between damaged and undamaged soybean nor between resistant and susceptible cultivars. In addition, the resistant cultivar Dowling and IAC100 showed consistency in the main volatile compounds induced: (*E*,*E*)-α-farnesene, methyl salicylate, (*Z*)-3-hexenyl acetate. Further laboratory studies are necessary to investigate the importance of these compounds for attraction of the egg parasitoid *T. podisi*. Once understood, these volatile compounds could help to develop new strategies for control of *E. heros* using natural enemies in soybean crops.

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Role of Volatile and Non-Volatile Plant Secondary Metabolites in Host Tree Selection by Christmas Beetles

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Abstract Individual *Eucalyptus* trees in south-eastern Australia vary considerably in susceptibility to herbivores. On the one hand, studies with insect herbivores have suggested that variation in the concentrations of foliar monoterpenes is related to variation in susceptibility. On the other, studies with marsupial folivores have suggested that variation in the concentrations of sideroxylonals (a group of formylated phloroglucinol compounds) is responsible for variation in susceptibility. We examined relative importance of sideroxylonals and 1,8-cineole (a dominant monoterpene) in host tree selection by Christmas beetles (*Anoplognathus* species: Coleoptera: Scarabaeidae) by using no-choice experiments, choice/no-choice experiments, and manipulative experiments in which concentrations of sideroxylonals or 1,8-cineole were altered. We used two species of host *Eucalyptus*, one species of non-host *Eucalyptus*, and three species of non-host non-*Eucalyptus* trees. Leaf consumption by Christmas beetles was negatively correlated with the concentrations of sideroxylonals and 1,8-cineole. Artificial increases in the concentration of sideroxylonals or 1,8-cineole reduced leaf consumption by Christmas beetles. An artificial reduction in foliar monoterpenes had no effect on leaf consumption by the beetles when leaves contained high or very low concentrations of sideroxylonals. However, when the concentration of sideroxylonals was moderate, a reduction in the foliar monoterpenes increased leaf consumption by the beetles. Therefore, monoterpenes such as 1,8-cineole may be used as a negative cue by Christmas beetles. The pattern of food consumption on non-host *Eucalyptus* species and non-host non-*Eucalyptus* species suggest that both positive and negative cues may be used by Christmas beetles to select host trees.

Key Words *Anoplognathus* · Cineole · *Eucalyptus melliodora* · *Eucalyptus polyanthemos* · *Eucalyptus sideroxylon* · Formylated phloroglucinol compounds · Plant-herbivore interactions · Sideroxylonals

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Introduction

Plant secondary metabolites play important roles in host selection by herbivores. Some of these compounds may act as positive (attractants) or negative cues (deterrents) that signal the quality of plants as food, while other compounds may act as toxins that reduce performance of insects. At least some specialist insect herbivores choose host plants upon which to oviposit on the basis of plant secondary metabolites (e.g., Steinbauer et al., 2004; Östrand et al., 2008), and many studies have documented negative

relationships between performance of herbivores and concentrations or types of secondary metabolites (e.g., Feeny, 1968; Matsuki and MacLean, 1994 and references therein; Osier et al., 2000). When specialist insect herbivores include a new host species, the new host species is likely to be taxonomically close to, or share at least some secondary metabolites with, the original host species (Futuyma and McCafferty, 1990; Janz and Nylin, 1998; Janz et al., 2001).

The importance of intra-specific variation in secondary metabolites to plant—herbivore interactions is widely accepted (Denno and McClure, 1983; Fritz and Simms, 1992; Hunter, 1997). For feeding or egg-laying, specialist insect herbivores must select not only the right host species but also the right individual of the host species.

Some *Eucalyptus* (Myrtaceae) species show striking intra-specific variation in concentrations of secondary metabolites and susceptibility to herbivores. For example, trees growing in close proximity may show marked variation in susceptibility to Christmas beetles (*Anoplognathus* species: Coleoptera: Scarabaeidae) (Edwards et al., 1993), and occasionally, there are some showing within tree variation in susceptibility. One branch of a particular study tree of *E. melliodora* suffered only 5% defoliation, while the rest of the tree suffered 85% defoliation (Edwards et al., 1990). There were negative correlations between herbivory by Christmas beetles and the proportion of 1,8-cineole (a monoterpene) in six species of *Eucalyptus* (Edwards et al., 1993). 1,8-Cineole and other components of essential oils in leaves of eucalypts are considered to play important defensive roles against other insect herbivores (e.g., Morrow and Fox, 1980; Ohmart and Larsson, 1989; Stone and Bacon, 1994; Southwell et al., 2003).

Studies of arboreal marsupial folivores on *Eucalyptus* have shown that consumption is negatively affected by a group of compounds called formylated phloroglucinol compounds (FPCs) (Pass et al., 1998; Lawler et al., 2000; Wallis et al., 2002; Moore et al., 2004a). Formylated phloroglucinol compounds are found only in some species in Myrtaceae, and leaves of many *Eucalyptus* species contain one to several different FPCs (Ghisalberti, 1996; Eschler et al., 2000).

Sideroxylonals (a class of FPCs) show strong antifeedant activity against marsupial folivores, and these animals can adjust food intake to regulate the amounts of sideroxylonals being ingested (Lawler et al., 1998; Wallis et al., 2002). Sideroxylonals appear to cause emetic responses in animals that have ingested the chemical (Lawler et al., 1998; DeGabriel et al., 2010). However, because sideroxylonals are not volatile, animals have to ingest some leaves to detect concentrations in foliage. It has been hypothesized that volatile monoterpenes might act as pre-ingestive cues to marsupial folivores (Lawler et al., 1999), and subsequently, it has been shown that concentrations of sideroxylonals and some monoterpenes are positively correlated (Moore et al., 2004b). Following studies with marsupial folivores, it was shown that performance of Christmas beetles and some Lepidopterans are negatively affected by sideroxylonals (Steinbauer and Matsuki, 2004; Andrew et al., 2007; Matsuki et al., 2010).

The purpose of this study was to examine roles of sideroxylonals and 1,8-cineole in host selection and food consumption by Christmas beetles. These beetles are particularly suited to examining roles of the two secondary metabolites. Some of the host species of Christmas beetles (*E. melliodora* and *E. sideroxylon*) show strong within-species variation in concentrations of both sideroxylonals and 1,8-cineole (Watson, 1998; Moore et al., 2004a). As noted, Christmas beetles show strong preference for some individuals of the host species over other individuals (Edwards et al., 1993; Andrew et al., 2007). They chew through leaves, and thus, must ingest sideroxylonals. In this study, laboratory experiments were carried out to examine effects of sideroxylonals and 1,8-cineole on food consumption by Christmas beetles.

Methods and Materials

Study Organisms We examined leaf consumption by 6 species of Christmas beetles that are commonly found in south-eastern Australia (*Anoplognathus chloropyrus*, *A. hirsutus*, *A. montanus*, *A. pallidicollis*, A. *suturalis*, and *A. viriditarsis*). Larvae feed on roots of grasses and crops. Adult beetles preferentially feed on young leaves of *Eucalyptus* that are nearly or completely expanded (Carne et al., 1974). Leaves of less than 3 yr-old also are eaten when young leaves are not available. In south-eastern Australia, Christmas beetles feed on at least 12 species of *Eucalyptus* (Edwards et al., 1993; M. Matsuki, pers. obs.).

We used *E. melliodora*, *E. polyanthemos*, and *E. sideroxylon*. Sideroxylonals (a mixture of three isomers A, B, and C) are the major class of FPCs found in these species (Eschler et al., 2000). *Eucalyptus melliodora* and *E. sideroxylon* are typical hosts of Christmas beetles, while leaves of *E. polyanthemos* trees are rarely eaten (M. Matsuki, pers. obs.; R. Andrew, pers. comm.). Cyanogenic glycosides are in leaves of *E. polyanthemos* but not in leaves of *E. melliodora* or *E. sideroxylon* (Gleadow et al., 2008). The three *Eucalyptus* species grow naturally in woodlands throughout south-eastern Australia and are similar in many aspects of their natural history (Boland et al., 1984). We also used *Betula* sp. (Betulaceae), *Quercus* sp. (Fagaceae), and *Populus tremuloides* (Salicaceae) as non-host species that are only distantly related to the host species. These are exotic ornamental trees in Australia and are not known to contain sideroxylonals or to store monoterpenes.

Choice of Trees Trees (m1–m3, s1–s3, & p1–p3) were chosen on the basis of variation in leaf consumption by marsupial folivores. Among 8 *E. melliodora* trees tested, leaf consumption by koala (*Phascolarctos cinereus*: Moore et al., 2004a) and common brushtail possum (*Trichosurus vulpecula*: Watson, 1998) was highest on m3, intermediate on m2, and lowest on m1. Among 12 *E. sideroxylon* trees tested, leaf consumption by common brushtail possum (Watson, 1998) and common ringtail possum (*Pseudocheirus peregrinus*: Lawler et al., 2000) was highest on s3, intermediate on s2, and lowest on s1. Among 36 *E. polyanthemos* trees tested, leaf consumption by common ringtail possum (Lawler et al., 2000) was highest on p3, intermediate on p2, and lowest on p1. We also chose some susceptible (m6, m7, s6, and s7) and resistant (m4, m5, s4, and s5) trees of *E. melliodora* (m4–m7) and *E. sideroxylon* (s4–s7) on the basis of the records of herbivory by Christmas beetles (mostly *A. montanus*) in the field (P. B. Edwards and W. J. Wanjura, unpubl.). These were some of the trees studied by Edwards et al. (1993). Trees s8–s11 (*E. sideroxylon*) had never been used in studies with vertebrates or insects. During the study period, the trees used in this study suffered only minor defoliation by herbivores. Trees m4–m7 were located in Yeoval, New South Wales (32°44′S, 148°39′E), and s4–s7 were located in Cumnock, New South Wales (32°55′S, 148°44′E). All other trees were located in Canberra, Australian Capital Territory (35°16′S, 149°7′E). The tree numbers indicate unique trees. For example, m1 indicates the same tree throughout this paper.

Physical and Chemical Characteristics of Leaves We measured leaf water content and specific leaf weight. *Eucalyptus* leaves contain volatile terpenes, and leaves were dried at 40°C for 2 d so as not to lose these compounds. Specific leaf weight was measured by weighing a small disk of a known area cut from the leaf blade.

In 1997–99, concentrations of monoterpenes were determined by gas chromatography (GC) using the method described in Edwards et al. (1993). Two leaves per tree were analyzed for monoterpenes. The concentration of sideroxylonals was determined by high performance liquid chromatography (HPLC) using the method described in Lawler et al. (2000). A mixture of the three isomers of sideroxylonal was extracted and purified as described elsewhere (Eschler and Foley, 1999). Concentrations of leaf nitrogen in 1997–98 and those of 1,8-cineole and sideroxylonals in 1999–2000 were estimated by using near-infrared reflectance spectroscopy, NIRS (Lawler et al., 2000).

The calibration equation for the concentration of leaf nitrogen was developed with a set of NIRS spectra and measured nitrogen content of 226 pre-existing samples of various *Eucalyptus* species by using a modified partial least square regression (ASTM 1995). We validated the calibration equation with 3 samples of *E. melliodora* and 6 samples of *E. sideroxylon* that were not used in the calibration equation. The calibration equations for sideroxylonals and 1,8-cineole were developed from a set of NIRS spectra and measured sideroxylonals and 1,8-cineole concentrations of 49 *E. melliodora* samples by using partial least square regressions. We used a cross-validation procedure (Infrasoft International, 1996; Brown, 1993) to validate calibration equations for total sideroxylonals and 1,8-cineole.

We classified leaves into three broad categories of concentrations of sideroxylonals or 1,8-cineole: low concentrations (<5 mg sideroxylonals or 1,8-cineole g^{-1} leaf dry matter); intermediate concentrations (5–20 mg sideroxylonals g^{-1} leaf dry matter or 5–10 mg 1,8-cineole g^{-1} leaf dry matter); and high concentrations (>20 mg sideroxylonals g^{-1} leaf dry matter or >10 mg 1,8-cineole g^{-1} leaf dry matter).

Consumption Trials We conducted five types of experiments in the three consecutive Austral summers between 1997 and 2000: (1) no-choice experiments; (2) choice/no-choice experiments; (3) experiments where the foliar concentration of sideroxylonals was enhanced; (4) an experiment where the foliar concentration of cineole was reduced via steaming; and (5) experiments where the foliar concentration of cineole was enhanced (see below for details).

For each experiment, several small branches were taken from each experimental tree so that leaves used were representative of leaves on experimental trees. Size and age of leaves in a given season were standardized visually in order to minimize these effects on leaf consumption. In the summer of 1997–1998, we used leaves of 1.5–2.5 yearr of age because leaves less than 1 yr-old were rare. However, the study trees produced new leaves in the autumn—spring of 1998, and by late December 1998, almost all old leaves had abscised. Therefore, we used leaves of 6- to 8-mo-old in the summer of 1998–1999. We used leaves that were approximately 1.5 yr-old in the summer of 1999–2000.

Adult beetles were collected in the field around Canberra. *Anoplognathus chloropyrus* also was collected from three other locations: Ginninderra, (35°09′S, 149°03′E); Gunning (34°47′S, 149°16′E); and Boorowa (34°26′S, 148°43′E), New South Wales.

To estimate the dry weight of leaf consumed by each beetle, we used two leaves for each insect. Each leaf was weighed and placed individually in clear plastic screw-top vials (250 ml) with moist plaster of Paris to maintain leaf turgor. At the beginning of an experiment, each beetle was weighed and placed in one of the two vials. Vials for each beetle were kept together during an experiment. Experiments were terminated after 5–15 h when some leaves were

nearly completely consumed. All experiments were started in the morning and terminated in the evening of the same day. Each beetle was weighed again at the end of each experiment. Each leaf was placed in a small envelope and dried at 40°C for 2 day. The water content of the leaf that had not been offered to insects (control leaf) was used to estimate the water content of the leaf that had been offered to an insect (trial leaf). The dry weight of leaf consumed by each insect was estimated as described elsewhere (Ayres and MacLean, 1987; Matsuki et al., 1994). There was no difference in relative consumption rates between male and female beetles for a given tree, and we used the pooled data for analyses.

No-choice and Choice/no-choice Experiments For no-choice and choice/no-choice tests, separate experiments were carried out for each *Eucalyptus* species, each leaf age, and each *Anoplognathus* species. In each no-choice experiment, the experimental design was 1 *Eucalyptus* species × 1 leaf age × 1 *Anoplognathus* species × 5 blocks × 3–4 treatments (trees) × 3 replicates. In choice/no-choice experiments, the experimental design was 1 *Eucalyptus* species × 1 leaf age × 1 *Anoplognathus* species × 5 blocks × 6 treatments (tree combinations) × 3 replicates. Blocks in the experiments represented rooms in three different buildings; however, the same five rooms were used in all experiments.

In no-choice experiments, one fresh leaf was placed in each vial. In choice/no-choice experiments, two fresh leaves were placed in each vial. For each *Eucalyptus* species, three trees were used in each choice/no-choice experiment: the susceptible, the resistant, and an intermediate tree (as defined above). We used all six pair-wise combinations of trees, i.e., susceptible-susceptible, susceptible-intermediate, susceptible-resistant, intermediate-intermediate, intermediate-resistant, and resistant-resistant. For example using *E. melliodora*, six pair-wise combinations of trees were m3-m3, m3-m2, m3-m1, m2-m2, m2-m1, and m1-m1, respectively.

We calculated relative consumption rates of leaves by Christmas beetles using a dry matter based method (Waldbauer, 1968). Using a subset of beetles, dry mass of beetles was estimated from the ratio of wet mass to dry mass for each beetle species/sex combination.

To examine preference of a tree i in choice/no-choice experiments, we used the index D (Jacobs, 1974):

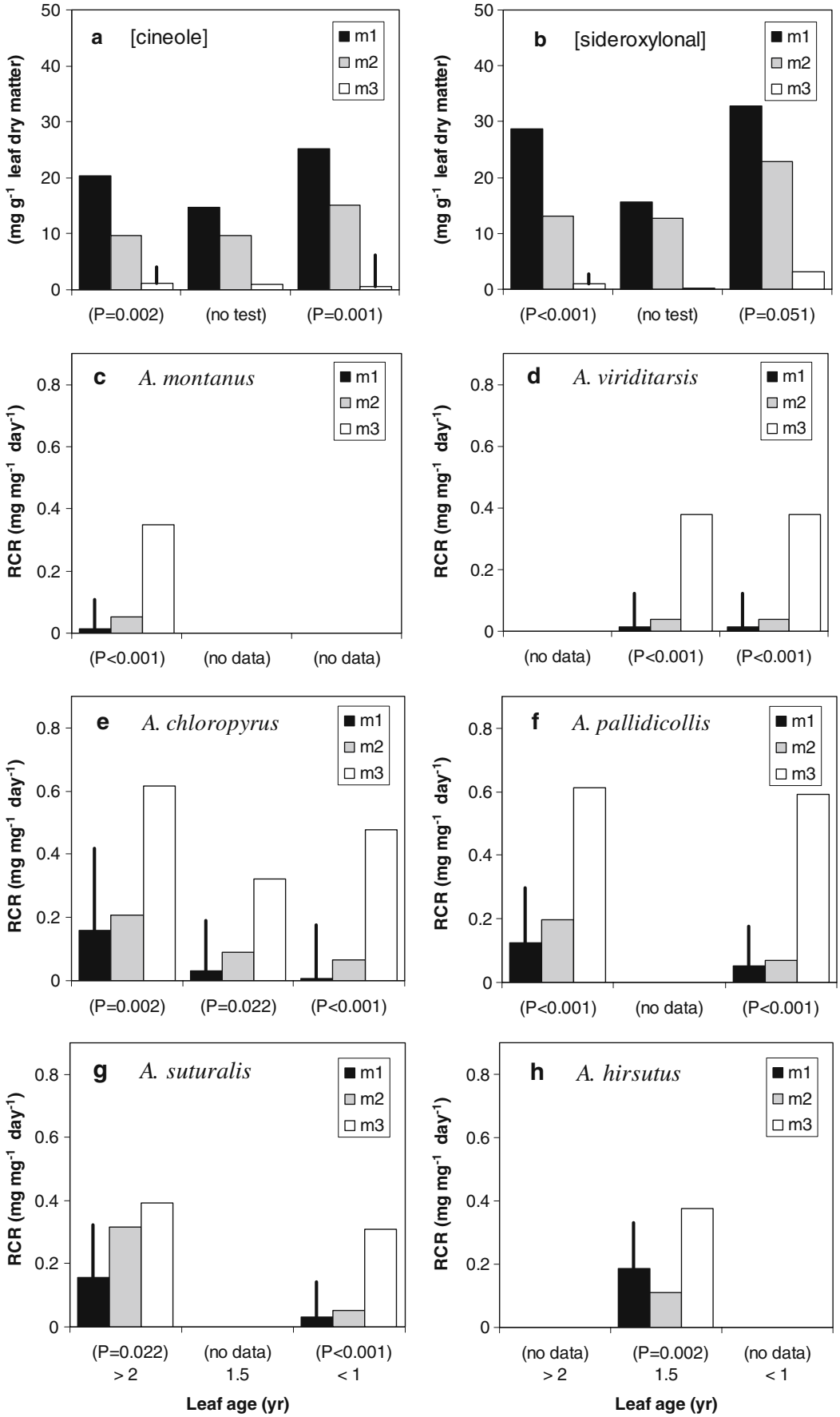
$$D_i = \frac{DR_i - RA_i}{RA_i + DR_i + 2RA_iDR_i},$$

Where DR_i is the dietary ratio, defined as the ratio between dry matter consumption of a leaf of tree i (DMC_i) and the total dry matter consumption of leaves from tree i and tree j (DMC_t): $DR_i=DMC_i/DMC_t$. RA_i is the relative amounts of leaf matter of tree i offered to a beetle during an experiment and is defined as the ratio between dry matter of a leaf of tree i offered (DMO_i) and the total dry matter of leaves of tree i and tree j (DMO_t): $RA_i=DMO_i/DMO_t$. Negative values of the index D indicate avoidance, while positive values indicate preference. For each experiment, the mean D_is were calculated, and 95% confidence intervals were used to detect whether the mean preference was significantly positive or negative. We used the index D because it met all three criteria for a good index of diet preference proposed by Cock (1978) and is sensitive to small changes in preference (Kam et al., 1997).

Manipulating the Foliar Concentration of Sideroxylonals We manipulated the foliar concentration of sideroxylonals by painting leaves with solutions of purified compounds. We used leaves of an *E. melliodora* tree that was most susceptible to marsupial folivores (m3) and another that was most resistant to marsupial folivores (m1). Purified sideroxylonals were dissolved in 10% methanol: 90% dichloromethane. We painted solutions of sideroxylonals onto leaves of m3 so that the treatment leaves would have approximately 20, 40, or 80 mg sideroxylonals per g leaf dry matter. In *E. sideroxylon* and *E. melliodora* in the field, only a small number of trees have greater than 40 mg sideroxylonals per g leaf dry matter (Watson, 1998; Wallis et al., 2002). We also painted leaves with solvent only as controls. Solutions of sideroxylonals were prepared in 3 different concentrations so that approximately the same amount of the solutions was applied to each leaf. The experimental design of the sideroxylonal manipulation experiments was 1 *Anoplognathus* species × 5 blocks × 7 treatments (2 trees × no solvent or solvent control + 3 concentrations of sideroxylonals) × 3 replicates. Blocks were as in no-choice and choice/no-choice experiments. Separate experiments were carried out for four *Anoplognathus* species.

Cineole Addition Experiments We also artificially increased the concentration of 1,8-cineole by painting leaves with purified compound. Approximately 0.1 ml of purified 1,8-cineole was applied to leaves of the most susceptible *E.*

Fig. 1 Mean concentrations of sideroxylonals and 1,8-cineole and mean relative consumption rates (RCR) of Christmas beetles. **a**–**h** *Eucalyptus melliodora* trees (m1–m3) and **i**–**p** *Eucalyptus sideroxylon* trees (s1–s3) with records of susceptibility to marsupial folivores. N=15 beetles per tree. *Grey bars* and *open bars* represent intermediate and susceptible trees, respectively, to marsupial folivores. *Solid bars* represent a resistant tree to marsupial folivores. The P-value for the differences among three trees for each leaf age is shown in parentheses. When $P<0.05$, the LSD for comparing the means is shown as the vertical line. No statistical test was carried out for concentrations of sideroxylonal and 1,8-cineole in 1.5 yr-old leaves because the concentration for each tree was estimated from ground material of multiple leaves per estimate using NIRS


a [cineole]
b [sideroxylonal]
(mg g-1 leaf dry matter)
m1
m2
m3
(P=0.002)
(no test)
(P=0.001)
(P<0.001)
(no test)
(P=0.051)
c A. montanus
d A. viriditarsis
e A. chloropyrus
f A. pallidicollis
g A. suturalis
h A. hirsutus
RCR (mg mg-1 day-1)
(P<0.001)
(no data)
(no data)
(P<0.001)
(P=0.002)
(P=0.022)
(P<0.001)
(P=0.022)
> 2
(no data)
1.5
(P<0.001)
< 1
(no data)
> 2
(P=0.002)
1.5
(no data)
< 1
Leaf age (yr)

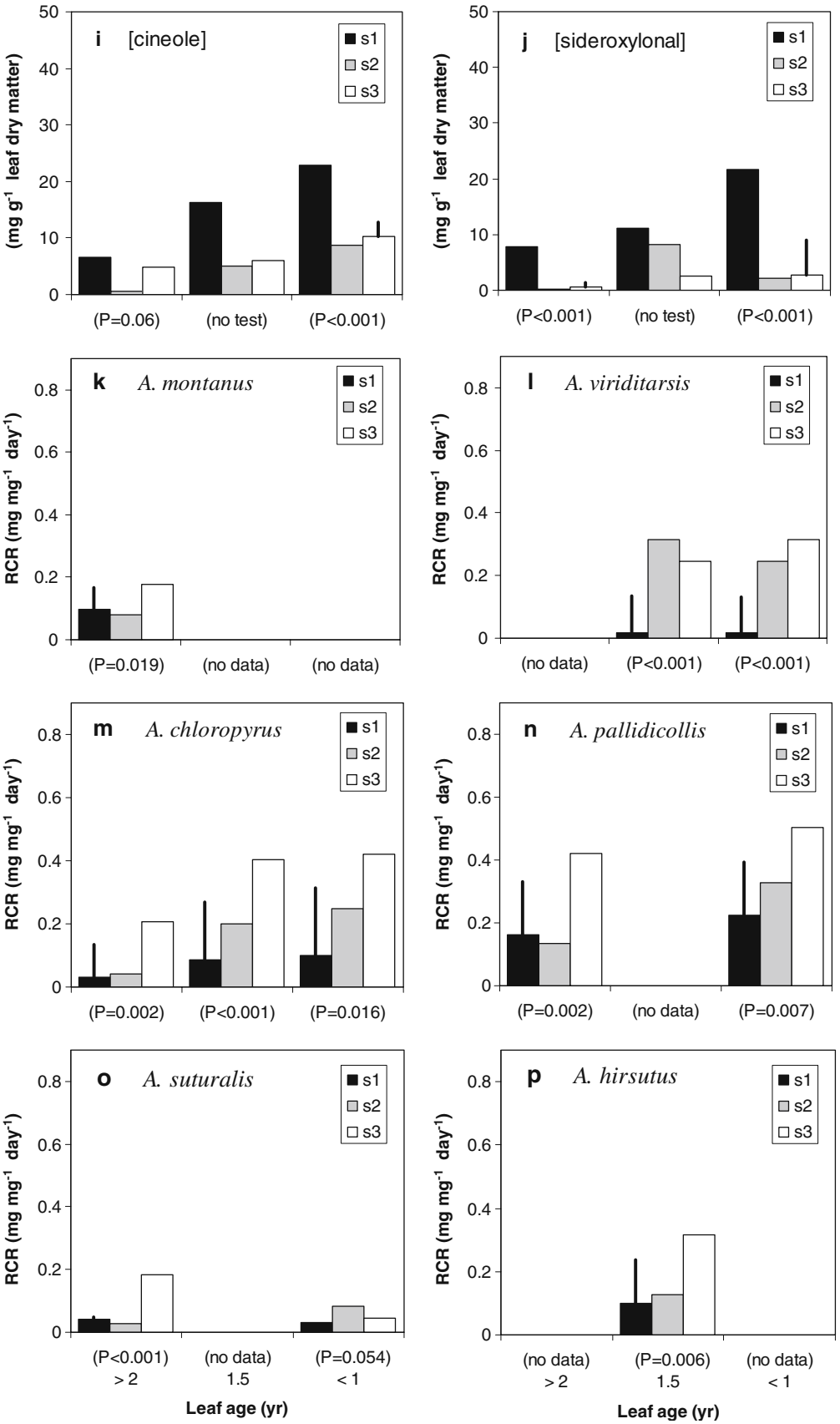


Fig. 1 (continued)

melliodora tree (m3). We used meshed screw-caps to avoid the build-up of vapor pressure inside the vials due to evaporation of 1,8-cineole. The design of the cineole addition experiments was 1 *Anoplognathus* species × 1 block × 2 treatments (cineole addition or control) × 1 tree × 10 replicates. Separate experiments were carried out for five *Anoplognathus* species.

Manipulating the Foliar Concentration of 1,8-Cineole by Steaming We reduced the foliar concentration of 1,8-cineole by placing leaves in a stream of steam. In this experiment, we used leaves of the two *E. melliodora* trees used in the sideroxylonal manipulation experiments (i.e., m1 and m3) and an *E. sideroxylon* tree (s9) with an intermediate concentration of sideroxylonals and a high concentration of 1,8-cineole. Leaves were steamed for 1 min. Fresh (i.e., unsteamed) leaves of the 3 trees were used as controls. Examination of the steamed leaves by GC revealed that steaming resulted in approximately 50% reduction in all monoterpenes, including 1,8-cineole (data not shown). The experimental design of the cineole reduction experiment was 5 blocks × 2 treatments (steaming or control) × 3 trees × 3 replicates. Blocks were as in no-choice and choice/no-choice experiments. This experiment was carried out with *A. chlorophyrus* only.

Experiment with Exotic Species To examine leaf consumption of species without sideroxylonals or monoterpenes, we used 3 species of exotic ornamental trees (*Betula* sp., *Quercus* sp., and *Populus tremuloides*) in a no-choice experiment. Leaves of the exotic species were collected from ornamental trees around the laboratory at CSIRO in Canberra. We included the most susceptible tree of *E. melliodora* (m3) as the standard in the experiment. The experimental design of the exotic species experiment was 5 blocks × 4 treatments (tree species) × 3 replicates. Blocks were as in no-choice and choice/no-choice experiments. This experiment was carried out with *A. chlorophyrus* only.

Statistical Analyses We analyzed data on leaf physical and chemical characteristics and consumption using ANOVAs (when the data set was balanced) or restricted maximum likelihood analyses (when the data set was unbalanced). When we examined differences among species, populations, and individual trees in leaf physical and chemical traits, we treated species, populations, and trees as fixed factors in nested ANOVAs. Since we used only 3 individuals of *E. polyanthemos* from 1 population in this study, we excluded *E. polyanthemos* from the analysis of differences between species.

In this study, we did not statistically examine effects of host plant population/beetle species on leaf consumption by beetles. The reasons for this are: (1) separate experiments were conducted for each beetle/host population combination; and (2) leaf consumption by beetles decreased gradually as the season progressed. Therefore, if a particular species of Christmas beetle shows lower consumption in an experiment conducted late in the season with a host population when compared with its consumption in an experiment conducted early in the season with another host population, the difference in consumption is likely due to combined effects of season and differences in host plant leaf chemistry.

However, we were able to examine relationships between the reduction in the mean relative consumption rates (RCRs) and changes in leaf traits, (specific leaf weight and concentrations of sideroxylonals and 1,8-cineole) in *E. melliodora* and *E. sideroxylon*. For each no-choice experiment, we calculated differences in the mean RCRs and the three leaf traits between a pair of trees: (1) resistant – susceptible; (2) resistant – intermediate; and (3) intermediate – susceptible. For each beetle species, the changes in the mean RCRs and leaf traits between pairs of trees from different experiments were pooled. *Anoplognathus montanus* and *A. chloropyrus* were the only species with more than five comparisons per tree pair, and other beetle species were not used in this analysis. For each beetle species and each of the three pairs of trees, we then examined the slope of the regressions between the changes in the mean RCRs (response variable) and the changes in each of the tree leaf traits (explanatory variable). Because the three leaf traits sometimes showed significant correlations (i.e., multicollinearity), we were not able to compare relative strength of the three leaf traits by using multiple regressions. We used GENSTAT (Genstat 5 Committee, 1993) and SAS (SAS Institute, 1989) for the statistical analyses.

Results

Host Eucalyptus Species Under no-choice situations, leaf consumption by all six species of Christmas beetles showed marked variation among three trees of *E. melliodora* (m1–m3) and three trees of *E. sideroxylon* (s1–s3), and all six species of beetles showed qualitatively consistent pattern of leaf consumption (Fig. 1). The Christmas beetles consumed more leaves from trees with markedly lower concentrations of sideroxylonals and 1,8-cineole than trees with high concentrations of sideroxylonals and 1,8-cineole. Leaves of the resistant trees (with high concentrations of sideroxylonals and 1,8-cineole) tended to contain higher concentrations of some other monoterpenes such as α-pinene, limonene, and terpineol but lower concentrations of *p*-cymene and α-phellandrene than leaves of susceptible trees (with low concentrations of sideroxylonals and 1,8-cineole) (Table 1).

Table 1 Differences in physical and chemical characteristics of leaves (>2 yr-old) among species, populations, and trees of *Eucalyptus melliodora* and *Eucalyptus sideroxylon* and differences between resistant and susceptible trees of the two *Eucalyptus* species

	Differences among			Resistant *vs.* susceptible		
	Species P^a	Populations P^a	trees P^a	P^b	Means	Unit
water content	0.20	0.41	**<0.001**	0.40	44.4 *vs.* 46.0	%
SLW[c]	0.23	0.87	**<0.001**	0.08	0.18 *vs.* 0.16	mg mm^{-2}
[nitrogen]	0.55	**0.02**	–	0.20	135 *vs.* 147	mg g^{-1}
[sideroxylonals]	0.25	0.25	**<0.001**	0.07	16.1 *vs.* 5.67	mg g^{-1}
[α-pinene]	0.07	**0.04**	**<0.001**	0.24	1.53 *vs.* 0.59	mg g^{-1}
[α-phellandrene]	0.92	0.49	**<0.001**	**0.02**	0.16 *vs.* 1.09	mg g^{-1}
[limonene]	0.14	0.96	**<0.001**	**0.007**	0.91 *vs.* 0.12	mg g^{-1}
[1,8-cineole]	0.73	0.72	**<0.001**	**0.003**	15.2 *vs.* 2.1	mg g^{-1}
[*p*-cymene]	0.44	0.96	**<0.001**	**0.007**	0.06 *vs.* 0.77	mg g^{-1}
[terpineol]	0.15	0.69	**<0.001**	0.06	0.88 *vs.* 0.05	mg g^{-1}

[a] from ANOVAs. $P<0.05$ are shown in bold

[b] from planned contrasts. $P<0.05$ are shown in bold

[c] SLW=Specific Leaf weight

However, 1,8-cineole was the dominant component of the *Eucalyptus* oils. The concentration of 1,8-cineole ranged from 0 to 27 mg g^{-1} leaf dry matter, while the maximum concentration of any one monoterpene other than 1,8-cineole was 3.5 mg g^{-1} leaf dry matter. Leaf nitrogen concentration, leaf water content, and specific leaf weight did not differ significantly between the resistant and susceptible trees (Table 1). When we examined relationships between changes in leaf consumption and changes in leaf traits between pairs of trees (resistant – susceptible, resistant – intermediate, and intermediate – susceptible), the decreases in relative consumption rates in *A. montanus* and *A. chloropyrus* tended to be more strongly associated with the increases in concentrations of sideroxylonals and 1,8-cineole than the changes in specific leaf weights (Table 2).

The same qualitative pattern of difference in leaf consumption of the same six trees (m1–m3 and s1–s3) was observed between years and between young (<1 yr-old) and old leaves (>2 yr-old). The same qualitative pattern also was observed in three different populations of *A. chloropyrus* (the only species examined) in the same year (Fig. 2). Moreover, for at least two species of Christmas beetles (*A. montanus* and *A. chloropyrus*) the same qualitative pattern of tree discrimination associated with the same pattern of leaf chemical composition was observed in trees from Yeoval (m4–m7) and Cumnock (s4–s7) (Fig. 3). For these trees, leaf consumption in the laboratory was correlated directly with the historical record of defoliation by Christmas beetles (mostly *A. montanus*).

There was a striking difference in the behavior of Christmas beetles depending on the leaves offered. The entire leaf, except mid vein and leaf margins, was consumed when leaves with low concentrations of sideroxylonals and 1,8-cineole were offered. In contrast, when leaves with high concentrations were offered, beetles either did not feed at all or took one to several

Table 2 Relationships between reduction in relative consumption rates (ΔRCRs) by *Anoplognathus montanus* and *Anoplognathus chloropyrus* and increases in concentrations of sideroxylonals (Δsideroxylonals) or 1,8-cineole (Δ1,8-cineole), or changes in specific leaf weight (ΔSLW)

Response variable	N^a	Explanatory variable		
ΔRCRs between the pair of trees shown below		Δsideroxylonals	Δ1,8-cineole	ΔSLW
A. montanus				
resistant – susceptible	8	−0.80±0.24[b] (**0.02**[c])	−0.66±0.31 (0.08)	−0.57±0.41 (0.22)
resistant – intermediate	6	0.55±0.43 (0.25)	−0.88±0.23 (**0.02**)	−0.12±0.50 (0.83)
intermediate - susceptible	6	−0.64±0.38 (0.17)	−0.97±0.13 (**0.002**)	−0.89±0.23 (**0.02**)
A. chloropyrus				
resistant – susceptible	7	−0.76±0.29 (**0.05**)	−0.92±0.17 (**0.003**)	−0.29±0.31 (0.54)
resistant – intermediate	8	0.10±0.14 (0.82)	−0.97±0.10 (**<0.001**)	−0.11±0.41 (0.79)
intermediate - susceptible	8	−0.66±0.31 (0.08)	−0.87±0.20 (**0.005**)	−0.58±0.33 (0.13)

[a] N=number of comparisons

[b] Standardized regression coefficient (= slope)±SE

[c] P-value for whether the slope=0. $P<0.05$ are shown in bold

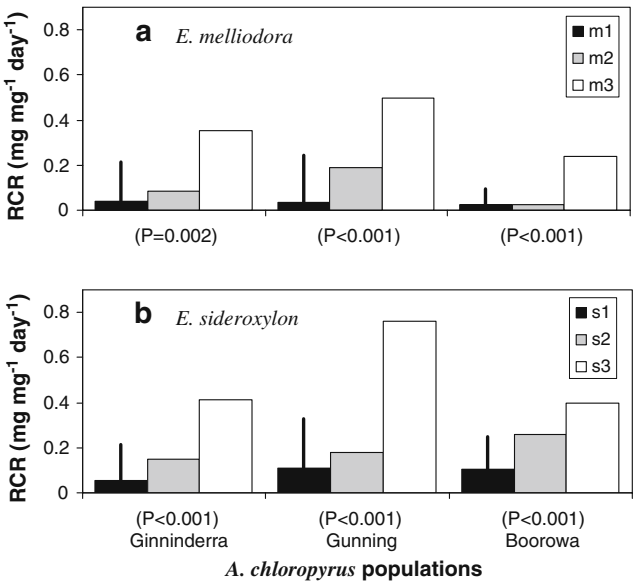


Fig. 2 Mean relative consumption rates (RCR) of three different populations of *Anoplognathus chloropyrus* on **a** *Eucalyptus melliodora* and on **b** *Eucalyptus sideroxylon* trees. N=12–15 beetles per tree. Ginninderra, Gunning, and Boorowa are locations from which the three populations have been collected. The P-value for the differences among three trees for each population is shown in parentheses. When $P<0.05$, the LSD for comparing the means is shown as the vertical line. 1.5 yr-old leaves were used in all experiments, and the concentrations of sideroxylonals and 1,8-cineole were shown in Fig. 1

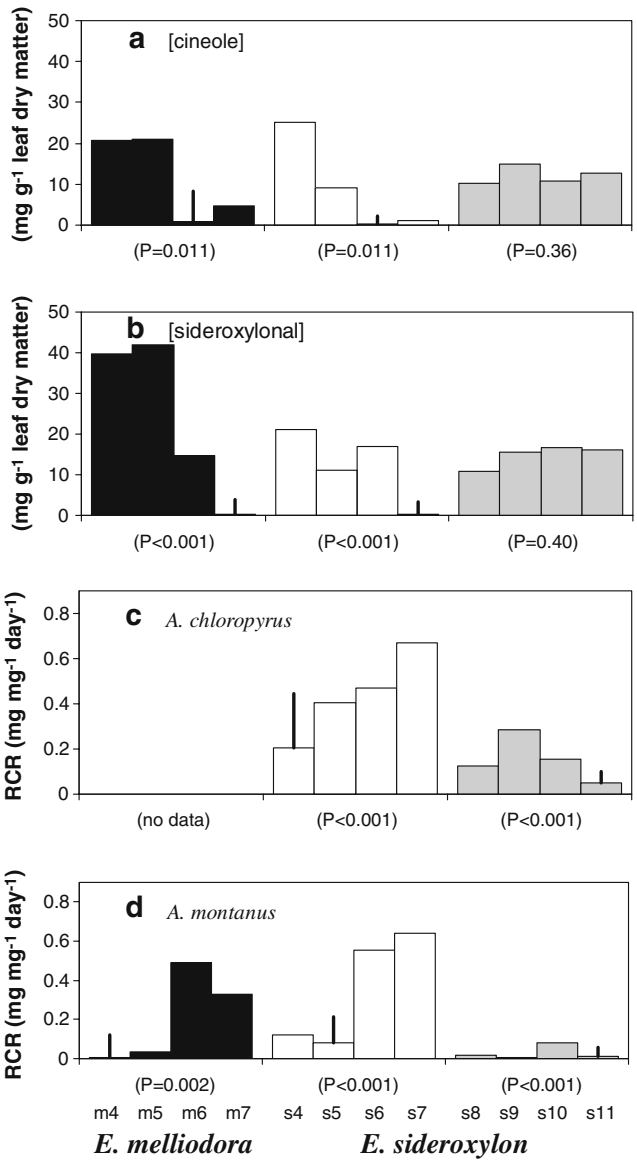


Fig. 3 Mean concentrations of **a** 1,8-cineole and **b** sideroxylonals of *Eucalyptus melliodora* trees from Yeoval (*solid bars*) and *Eucalyptus sideroxylon* trees from Cumnock (*open bars*) with records of herbivory by Christmas beetles and *Eucalyptus sideroxylon* trees from Canberra (*grey bars*) without records of herbivory and mean relative consumption rates (RCR) of **c** *Anoplognathus chloropyrus* and **d** *Anoplognathus montanus*. N=15 beetles per tree. The P-value for the differences among four trees for each population is shown in parentheses. When $P<0.05$, the LSD for comparing the means is shown as the vertical line. 1.5–2 yr-old leaves were used in all experiments

bites at a number of locations along the leaf margins but never continued feeding. Leaves of some trees (e.g., s9) either were consumed nearly entirely (as leaves of the susceptible trees) or not touched at all. Leaves of s9 contained an intermediate concentration of sideroxylonals and a high concentration of 1,8-cineole (Fig. 3). Some leaves of s9 were never chewed. These observations lead to the hypothesis that trees with low to moderate concentrations of sideroxylonals might be avoided by Christmas beetles due to presence of strong negative cues (e.g., high concentrations of 1,8-cineole). This hypothesis was tested by artificially manipulating the concentrations of sideroxylonals and 1,8-cineole (see below).

When beetles were given a choice between leaves from two different trees of *E. melliodora*, they always preferred the susceptible to the resistant or the intermediate tree (Table 3). The intermediate tree always was preferred to the resistant tree. When leaves from two trees of *E. sideroxylon* were offered together, beetles also always preferred the susceptible to the resistant or the intermediate tree (Table 3). The intermediate tree was preferred to the resistant tree by three species of Christmas beetles, but not by *A. chloropyrus*. Differences in preference among the three *E. sideroxylon* trees were not as large as those among the three *E. melliodora* trees.

Non-host Eucalyptus Species Leaf water content and specific leaf weight of *E. polyanthemos*, which was not a preferred host of Christmas beetles, did not differ from those of either *E. melliodora* or *E. sideroxylon* (data not shown). In terms of leaf chemistry, *E. polyanthemos* differed from the other two species by having generally low values and little between-individual variation in

Table 3 Between-tree feeding preference by *Anoplognathus* species with three species of *Eucalyptus* species

Trees compared	*E. melliodora*[a]	*E. sideroxylon*[a]	*E. polyanthemos*[b]
susceptible over intermediate	**0.43**[c,d] (15[e]) – **0.96** (15)	**0.43** (13) – **0.77** (13)	0.34 (10) – 0.56 (5)
susceptible over resistant	**0.41** (14) – **0.94** (14)	0.26 (15) – **0.49** (12)	−0.05 (10) – **1.04** (5)
intermediate over resistant	**0.42** (15) – **0.63** (14)	−0.81 (3) – **0.96** (5)	0.52 (10) – **0.99** (6)

All leaves used in choice/no-choice experiments were >2 yr-old

[a] experiments using *Anoplognathus montanus*, *Anoplognathus chloropyrus*, *Anoplognathus pallidicollis*, and *Anplognathus suturalis*

[b] experiments using *Anoplognathus montanus* and *Anoplognathus chloropyrus* only

[c] Ranges of mean values of the index D. When D>0, preference of trees as in the "Trees compared" column. When D<0, reversal in preference of trees from that in the "Trees compared" column

[d] Bold-faced numbers indicate values significantly greater than 0 at α=0.05

[e] Sample size

concentrations of α-phellandrene (0.19–0.53 mg g^{-1} leaf dry matter) and *p*-cymene (0 mg g^{-1} leaf dry matter). In contrast, like *E. melliodora* and *E. sideroxylon*, concentrations of sideroxylonals and 1,8-cineole in the three *E. polyanthemos* trees differed markedly (Fig. 4). Leaf consumption by Christmas beetles, however, did not show corresponding differences (Fig. 4). *Anoplognathus suturalis* consumed as much leaf material of *E. polyanthemos* as that of *E. melliodora* and *E. sideroxylon*, while five other species of Christmas beetles consumed much less leaf material of *E. polyanthemos* compared with the other *Eucalyptus* species.

Effects of Sideroxylonals and 1,8-Cineole on Leaf Consumption There was a negative dosage-dependent effect of sideroxylonals on leaf consumption in all four species of Christmas beetles tested (Fig. 5). Relative consumption rates (RCR) were reduced to approximately one third of those in the control when concentrations of sideroxylonals were high (40 and 80 mg g^{-1} leaf dry matter). In *A. chloropyrus* and *A. pallidicollis*, there was no effect of increased concentrations of sideroxylonals at least up to one half of the maximum concentration found naturally in leaves of *E. sideroxylon* and *E. melliodora* (i.e., 20 mg g^{-1} leaf dry matter).

When pure 1,8-cineole was painted onto leaves of *Eucalyptus* branches, Christmas beetles immediately ceased feeding and quickly moved far away from the painted 1,8-cineole. An artificial increase in the concentration of 1,8-cineole reduced leaf consumption by all five species of Christmas beetles tested (Fig. 5). The beetles did not feed on leaves painted with 1,8-cineole until the 1,8-cineole had evaporated. Once that happened, the treatment leaves were consumed just as the control leaves. Therefore, the observed reduction in consumption is due to effective reduction in feeding time caused by evaporating 1,8-cineole.

An artificial 50% reduction in the concentration of 1,8-cineole by steaming leaves increased leaf consumption by *A. chloropyrus* on s9 with the moderate natural concentration of sideroxylonals and high natural concentration of 1,8-cineole (Fig. 6: see s9 in Fig. 3a and b for natural concentrations of sideroxylonal and 1,8-cineole). However, steaming leaves did not affect the consumption of leaves when the natural concentrations of both sideroxylonals and 1,8-cineole were high (m1) or low (m3).

Exotic Species The mean relative consumption rates (RCRs) by *A. chloropyrus* varied among species of exotic ornamental trees: very low in *Populus tremuloides* (0.014 mg mg^{-1} day^{-1}) and moderately high in *Betula* sp. (0.20 mg mg^{-1} day^{-1}) and *Quercus* sp. (0.32 mg mg^{-1} day^{-1}). The mean RCR on *Quercus* sp. was not different from the mean RCR on m3 (0.45 mg mg^{-1} day^{-1}) (LSD=0.14 for comparing the means). Monoterpenes were not detected in leaves of these species.

Discussion

New findings from this study are: (i) high concentrations of sideroxylonals or 1,8-cineole alone reduce leaf consumption by Christmas beetles, and (ii) sideroxylonals may act as post-ingestive deterrents, while 1,8-cineole may act as a pre-ingestive deterrent in host selection by Christmas beetles. Consistent with previous studies, we also found that: (i) there were positive correlations between concentrations of 1,8-cineole and sideroxylonals in *Eucalyptus melliodora* (as in Moore et al., 2004a), *E. sideroxylon*, and *E. polyanthemos*, and (ii) trees with low concentrations of sideroxylonals (as in Andrew et al., 2007) and 1,8-cineole (as in Edwards et al., 1990, 1993) suffered greater leaf consumption by beetles than trees with high concentrations

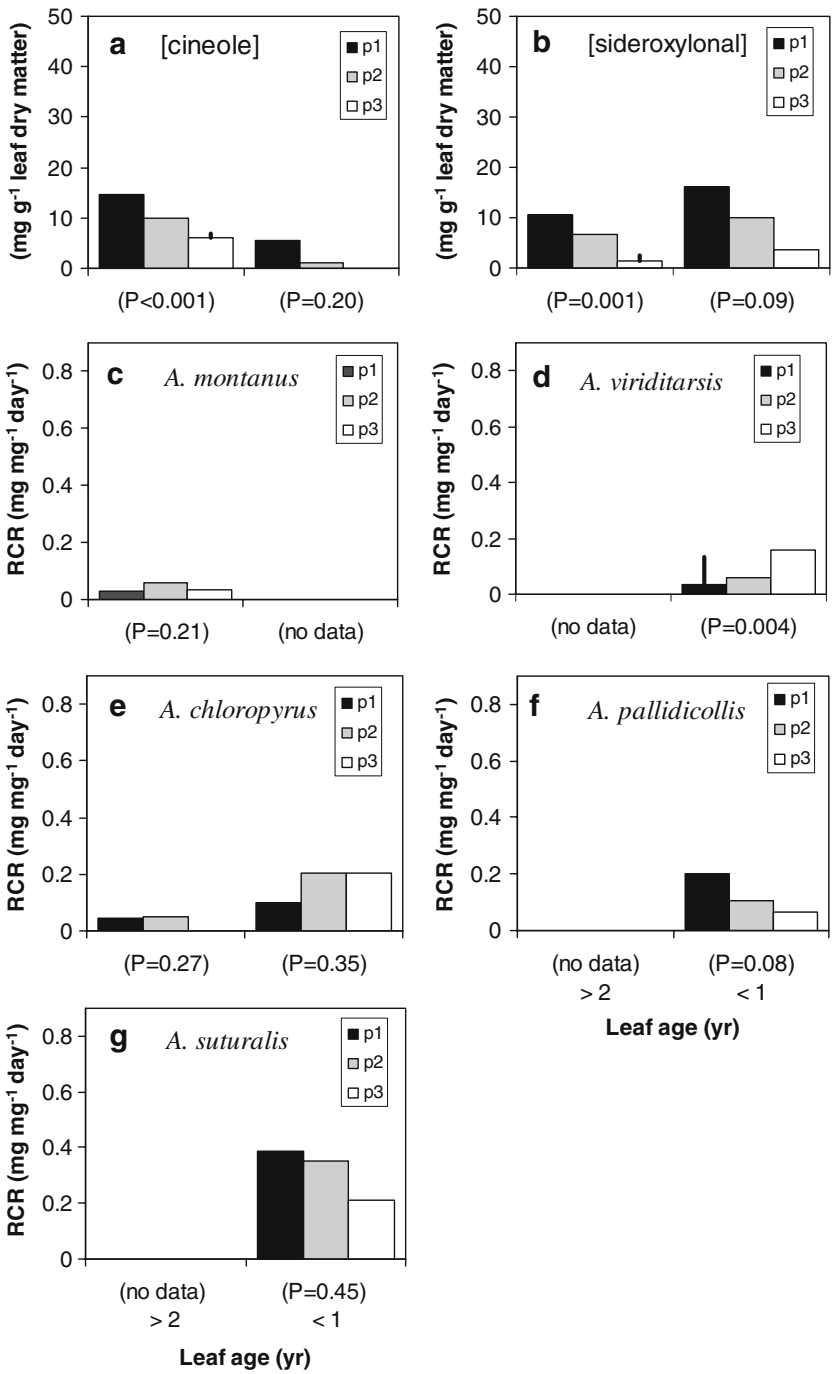


Fig. 4 Mean concentrations of **a** 1,8-cineole and **b** sideroxylonals of *Eucalyptus polyanthemos* trees and **c**–**g** mean relative consumption rates (RCR) of Christmas beetles. N=15 beetles per tree. The P-value for the differences among three trees for each leaf age is shown in parentheses. When $P<0.05$, the LSD for comparing the means is shown as the vertical line. *Solid bars* represent a resistant tree to common ringtail possum. *Grey bars* and *open bars* represent intermediate and susceptible trees, respectively, to common ringtail possums

of the two metabolites. Andrew et al. (2007) suggested that high concentrations of sideroxylonals conferred resistance of some individual trees of *E. melliodora* to Christmas beetles. However, by examining and manipulating multiple compounds in host and non-host species, we found that beetles might select host trees by detecting more than one plant secondary metabolite.

When leaves with high concentrations of sideroxylonals were offered, Christmas beetles took only one to several bites that were spread along the margin of leaves (M. Matsuki, pers. obs.). Thus, they appeared to detect the presence of sideroxylonals using sensory organs in their mouthparts. However, when leaves with moderate concentrations were offered, beetles continued feeding for some time but never consumed entire leaves. Thus, it appears that, even when sideroxylonals are ingested at low concentrations, there is another mechanism which stops Christmas beetles from ingesting large amounts of sideroxylonals. Marsupial folivores also regulate intake of sideroxylonals and some other FPCs (Lawler et al., 1998; Stapley et al., 2000; Wallis et al., 2002).

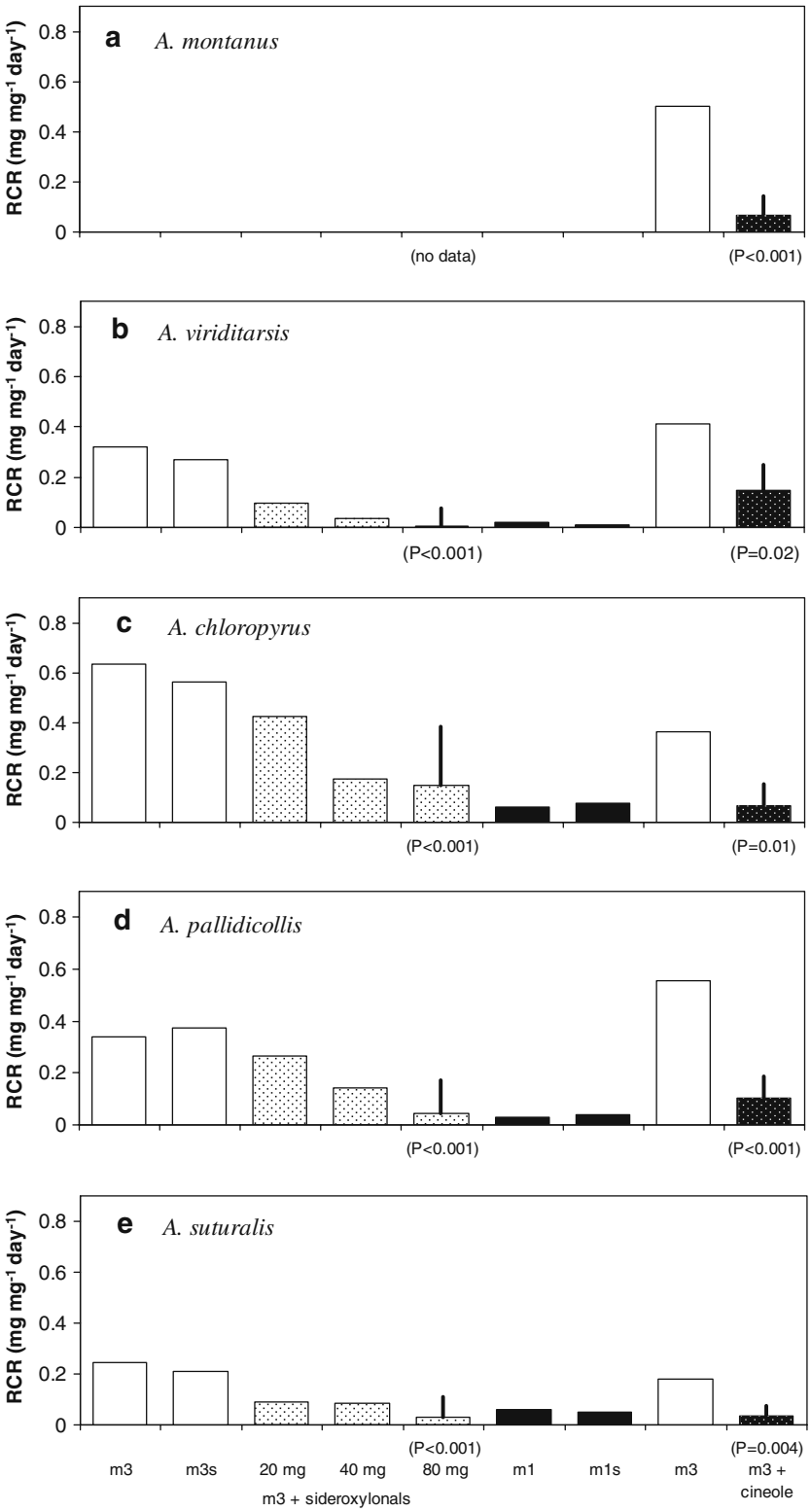


Fig. 5 Effects of artificially increased concentration of sideroxylonals and 1,8-cineole on relative consumption rate (RCR) of *Eucalyptus melliodora* leaves by five species of Christmas beetles. Data present means of $N=11–15$ beetles per tree. The P-value for the differences among the treatment is shown in parentheses. When $P<0.05$, the LSD for comparing the means is shown as the vertical line. Separate P-values and LSDs are shown for the sideroxylonal addition experiement (*seven bars on the left*) and the cineole addition experiment (*two bars on the right*). *Open bars* represent the susceptible tree (m3), and *closed bars* represent the resistant tree (m1). Solvent controls are represented as m1s and m3s. Trees m1 and m3 were also shown in Figs. 1, 2 and 6. > 2 yr-old leaves were used with *Anoplognathus chloropyrus* and *Anoplognathus pallidicollis,* while <1 yr-old leaves were used with *Anoplognathus montanus, Anoplognathus viriditarsis,* and *Anoplognathus suturalis*

Monoterpenes (e.g., 1,8-cineole) also can have harmful effects on insects post-ingestively (Rose, 1985). However, *A. montanus* and *Paropsis atomaria* (Coleoptera: Chrysomelidae) feeding on *Eucalyptus* leaves may simply "tolerate" (Morrow and Fox, 1980) or "avoid" (Edwards et al., 1993) monoterpenes.

Results of the experiment with steamed leaves suggest that Christmas beetles may use 1,8-cineole, or other volatile

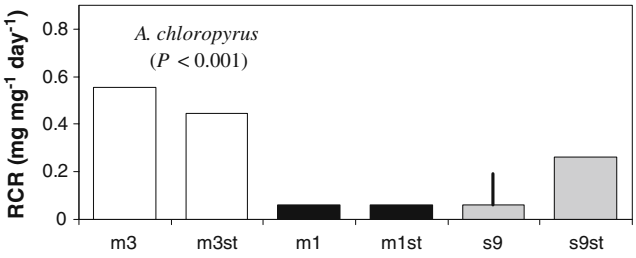


Fig. 6 Effects of artificially decreased concentration of 1,8-cineole by steaming leaves on relative consumption rate (RCR) of *Eucalyptus* leaves by *Anoplognathus chloropyrus*. Data present means of $N=15$ beetles per tree. The *P*-value for the differences among the treatment is shown in parentheses. The LSD for comparing the means is shown as the vertical line. *Open bars* represent the susceptible tree (m3), and the *closed bars* represent the resistant tree (m1). Trees m1 and m3 were also shown in Figs. 1, 2 and 5. Leaves of tree s9 (*grey bars*) contain high concentration of 1,8-cineole but only moderate concentration of sideroxylonals (cf. Fig. 3a & b). Leaf consumption on steamed leaves is indicated as m3st, m1st and s9st. >2 yr-old leaves were used in the experiment

compounds in *Eucalyptus* oils, as a pre-ingestive deterrent for selecting host trees. In the field, Christmas beetles fly around the canopy at close range. If a tree had high concentrations of sideroxylonals and 1,8-cineole, many beetles flew away without landing on a leaf or landed on a leaf but took off without feeding (M. Matsuki, pers. obs.). Since Christmas beetles seem to detect high concentrations of sideroxylonals with sensory organs in their mouthparts, the observed host selection behavior in the field is not likely driven by sideroxylonals in leaves. Christmas beetles may detect high concentrations of volatile terpenes in the atmosphere immediately surrounding resistant trees.

Guilford and Cuthill (1991) have suggested that evolution of unpalatability is not a likely result of selection on individuals that share the same phenotype (i.e., synergistic selection) because, unlike aposematic coloration in animals, palatable and unpalatable plants appear identical except for the difference in palatability. However, volatile compounds such as 1,8-cineole may act as a signal for the presence and the concentration of compounds such as sideroxylonals (this study; Lawler et al., 1999: for the debate on whether one plant secondary metabolite can serve as a signal for another, see Dethier and Post, 1979; Eisner and Grant, 1981; Launchbaugh and Provenza, 1993; Augner, 1994; Augner and Bernays, 1998).

Tuomi and Augner (1993) and Leimar and Tuomi (1998) showed that evolution of plant unpalatability might result from synergistic selection, even without a cue, if herbivores took samples from a population of plants and made generalizations about the population. *Eucalyptus melliodora* and *E. sideroxylon* showed striking variation in leaf chemistry among neighboring individuals (Edwards et al. 1993). Therefore, if synergistic selection has been involved in evolution of chemical defenses using sideroxylonals, the presence of volatile compounds such as 1,8-cineole, which can be used as a cue (Guilford and Cuthill 1991), is a more likely mechanism than leaf sampling and subsequent generalization by herbivores (Tuomi and Augner 1993; Leimar and Tuomi 1998).

An interesting twist in the *Eucalyptus*—herbivore system is that some trees of *E. sideroxylon* seem to have only low to intermediate concentrations of sideroxylonals but high concentrations of 1,8-cineole (e.g., s8–s11 in this study). If these trees suffer low levels of herbivory in the field, as is observed in the laboratory experiments, then it may be possible that high concentrations of 1,8-cineole in these trees act as a false signal.

So far, we have focused on the role of sideroxylonals and 1,8-cineole as negative cues in host selection, but host selection by Christmas beetles may involve negative and positive cues. Under natural conditions, Christmas beetles encounter four types of plants: (1) host *Eucalyptus* species that contain sideroxylonals and monoterpenes; (2) non-host *Eucalyptus* species that also contain sideroxylonals and monoterpenes; (3) non-host species that contain monoterpenes (but no sideroxylonals); and (4) non-host species that do not contain sideroxylonals or monoterpenes. Moreover, within the host *Eucalyptus* species, only a small proportion of individuals contain low concentrations of sideroxylonals (Lawler et al., 2000; Wallis et al., 2002). If negative cues (i.e., high concentrations of sideroxylonals and 1,8-cineole) alone were used to locate a small number of suitable host trees in the field, Christmas beetles may have to spend great amounts of time and energy before starting to feed.

Christmas beetles readily consumed leaves of some exotic species in captivity. Yet, they rarely consume leaves of these exotic plant species in the field. Leaves of the exotic species do not contain sideroxylonals or 1,8-cineole. Therefore, lack of feeding by beetles on those exotic species under natural conditions cannot be explained by high concentrations of sideroxylonals or 1,8-cineole in the leaves. Christmas beetles did not feed on *Populus tremuloides*. This is possibly because *P. tremuloides* have phenolic glycosides in their leaves (e.g., Osier et al., 2000).

Steinbauer and Wanjura (2002) suggested that Christmas beetles mistook *Schinus molle* (Anacardiaceae) for eucalypts because leaves of this exotic species contained monoterpenes. Other specialist insect herbivores also are attracted to non-host species that have similar secondary metabolites to the hosts (e.g., Murphy and Freeland, 2006; Östrand et al., 2008). However, presence of monoterpenes in general does not seem to signal the suitability as host plants because many *Eucalyptus* species are non-hosts for Christmas beetles. For example, leaves of most trees of *E. polyanthemos* are not consumed by Christmas beetles in the field even when beetles consume leaves on trees of *E.*

melliodora and *E. sideroxylon* in the same area (M. Matsuki, pers. obs.). Since three species of Christmas beetles consumed moderate amounts of *E. polyanthemos* leaves in captivity, it is more likely that *E. polyanthemos* leaves lack positive cues than that the leaves have negative cues. Concentrations of α-phellandrene and *p*-cymene generally were low and showed little between-individual variation in *E. polyanthemos*. In contrast, concentrations of these monoterpenes are negatively correlated with concentrations of 1,8-cineole in *E. sideroxylon and E. melliodora*. Thus, Christmas beetles may be using α-phellandrene or *p*-cymene as a positive cue for host plants. In noctuid moth species, positive and negative cues in a blend of odor are distinguished and processed separately in the brain (Vickers et al., 1998). Thus, to select trees, Christmas beetles may use both positive and negative cues. Alternatively, the ratio of various monoterpenes may be used to select host species by Christmas beetles (Edwards et al., 1993).

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Leaf Chemistry and Foliage Avoidance by the Thrips *Frankliniella occidentalis* and *Heliothrips haemorrhoidalis* in Glasshouse Collections

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Abstract Observational studies on foliage avoidance by the polyphagous thrips species *Frankliniella occidentalis* (Pergande) and *Heliothrips haemorrhoidalis* (Bouché) (Thysanoptera: Thripidae) identified six non-host species (*Allagopappus dichotomus* (Asteraceae), *Gardenia posoquerioides* (Rubiaceae), *Plectranthus aff. barbatus*, *Plectranthus strigosus*, *Plectranthus zuluensis* (Lamiaceae), and *Sclerochiton harveyanus* (Acanthaceae) among plants growing within a major glasshouse botanical collection. The effects of sequentially obtained acetone and aqueous methanol leaf extracts on mortality in first instar *Frankliniella occidentalis* were assessed. The acetone leaf extract of *Sclerochiton harveyanus,* which had the highest activity against the thrips, yielded four new iridoids, sclerochitonosides A–C, and sclerochitonoside B 4′-methyl ether. Mortality of *F. occidentalis* was increased on exposure to all four iridoids, and the most active iridoid was sclerochitonoside A (8-epiloganic acid 4′-hydroxyphenylethyl ester). Choice experiments demonstrated that this compound did not significantly deter *H. haemorrhoidalis* from treated leaf surfaces. The significance of iridoids in the defense mechanism of plants against thrips is discussed.

Key Words *Frankliniella occidentalis* · Glasshouse thrips · *Heliothrips haemorrhoidalis* · Iridoids · Plant defense · Plant resistance · *Sclerochiton harveyanus* · Western flower thrips

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Introduction

Rapid expansion in the distribution range of several invasive species of thrips and their associated tospoviruses has contributed to pressure on agricultural economies and food production in both developed and developing countries (Lewis, 1997a). The polyphagous nature of these invading species has expanded plant host records at a rate that currently exceeds the accumulation of knowledge on host selection, the efficacy of current biological and chemical controls for specific crop plants, and the development of new control agents or control strategies that aim to eliminate or contain emerging populations. Western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is a key species that continues to elude the controls available for use on protected crops in North America, Europe, Australia, Asia, and Africa. The success of *F. occidentalis* as a pest species has been attributed to a range of factors, from human assisted transmission to pesticide resistance (Kirk and Terry, 2003), in conjunction with its ability to feed and rapidly reproduce on a wide variety of hosts (Lewis, 1997b). Damage can be extensive because feeding and oviposition on young leaves, buds, and flowers causes leaf and petal discoloration, desiccation, and distortion of developing fruit. In addition, *F. occidentalis* is a vector for several species of tospoviruses such as tomato spotted wilt virus (TSWV) and impatiens necrotic spot virus (INVS) (Ullman et al., 1997, 2002). Another species; *Heliothrips haemorrhoidalis* (Bouché) (Thysanoptera: Thripidae), is a pest of a wide range of plantation crops such as avocado, tea, and citrus (Goodall et al., 1987; Holt, 1989; Dennill and Erasmus, 1992), and also damages ornamental crops and botanical collections held under glass in temperate regions. The pest status of this species is exacerbated by the fact that it reproduces parthenogeneti-

cally resulting in rapid fluctuations in populations over a relatively short period of time when conditions are optimal. Unlike *F. occidentalis*, *H. haemorrhoidalis* tend to feed on older leaves and do not transmit tospoviruses, although the high numbers achieved on foliage can cause complete defoliation of some crop species.

Frankliniella occidentalis and *H. haemorrhoidalis* discriminate among species of plants when selecting suitable hosts for feeding and reproduction from the diverse botanic collections held in the Temperate House, Royal Botanic Gardens, Kew, UK (Scott Brown, 2002). Variations in plant and foliage morphology, flower/foliage color and hue, volatile constituents and leaf chemistry of cultivated hosts influence the thrips. However, factors that prevent thrips from infesting certain species of plants from exotic glasshouse collections have not been widely reported. Plants from the Temperate House collections that both species of thrips avoid belong to families such as Acanthaceae, Asteraceae, Hyacinthaceae, Lamiaceae, Malvaceae, and Rubiaceae. Previous studies by Scott Brown (2002) examined the performance and preference of *H. haemorrhoidalis* on cut leaves of several of the non-hosts from the Temperate House under laboratory conditions and observed varying levels of disruption to the development and longevity of this insect. *Sclerochiton harveyanus* Nees (Acanthaceae), a large (>2 m) evergreen shrub native to southeastern Africa (Hyde and Wursten, 2010), appeared to be highly toxic to *H. haemorrhoidalis* (Scott Brown, 2002).

Plant secondary metabolites apparently influence the host selection behavior of polyphagous thrips (Mollema and Cole, 1996; Kim et al., 1998; Koschier et al., 2000, 2002), as does the availability of nutrients such as carbon and nitrogen (Bernays and Chapman, 1994; Brodbeck et al., 2001; Scott Brown et al., 2002). However, there is little relevant chemical data on the role of specific metabolites in determining whether plants are hosts or non-hosts for *F. occidentalis* and *H. haemorrhoidalis*.

The objectives of this study were to examine the relationships, if any, between the non-host plants in the glasshouses at the Royal Botanic Gardens (RBG), Kew and chemical constituents present in their foliage. Whole-leaf extracts from six species of non-host plants were toxic to *F. occidentalis*. Extracts that were most toxic to thrips were fractionated in an attempt to isolate and identify the active components.

Methods and Materials

Plant Material and Thrips Non-host plant species selected for these investigations were i) observed to remain free from thrips damage during monitoring studies carried out between 1994 and 2003 in the Temperate House, RBG, Kew, and ii) unable to support thrips on excised leaves under laboratory conditions (Scott Brown, 2002). Leaves from 6 non-host species of plants were collected from the Kew Temperate House, in 2004 (Table 1) and freeze-dried immediately. First instar *F. occidentalis* were collected from a stock culture reared on pods of *Phaseolus vulgaris* L. on a thin layer of vermiculite in sealed plastic containers (23 L× 12 W×7.5 H mm) (Stewart Plastics, UK). Cultures were reared in an incubator maintained at 25°C ± 1°C, with a photoperiod of L16:D8 hr. Adult and pupal stages of *H. haemorrhoidalis* were collected from infestations on *Hibbertia scandens* (Willd.) Dryand. (Dilleniaceae) in the Temperate House.

Preparation of Leaf Extracts Freeze dried leaf material was ground with mortar and pestle into a fine powder and extracted with acetone (100 ml per 10 g leaf powder) for 24 hr before filtering through Whatman 1 filter paper. Leaf residue was re-extracted in aqueous methanol (methanol: water, 80:20, v/v; 100 ml per 10 g leaf residue) for 48 hr and filtered. Aliquots (1 ml) of the acetone and aqueous methanol extracts were filtered through nylon acrodisc filters prior to analytical HPLC. The remaining extracts were taken to dryness by rotary evaporation (aqueous methanol extracts) or under nitrogen (acetone extracts).

HPLC Analysis of Leaf Extracts HPLC coupled to diode array detection was carried out using a Waters system (LC600 pump and controller, 996 photodiode array detector and 717 Plus autosampler) with a Merck LiChrospher 100RP-18 column (250 mm×4.0 mm i.d.; 5 μm particle size) operating at 30°C and a flow rate of 1.0 ml/min. The gradient elution program was based on A = methanol:acetic

Table 1 Six species of plants selected from the Temperate House (RBG, Kew) collection because they remained free from infestations of *Frankliniella occidentalis* or *Heliothrips haemorrhoidalis*

Species	Family	Kew Accession No.
Allagopappus dichotomus Cass.	Asteraceae	1988-4128
Gardenia posoquerioides S.Moore	Rubiaceae	1979-5490
Plectranthus aff. *barbatus* Andrews	Lamiaceae	1992-2021
Plectranthus strigosus Benth. ex E.Mey.	Lamiaceae	1967-41108
Plectranthus zuluensis T.Cooke	Lamiaceae	1992-2850
Sclerochiton harveyanus Nees	Acanthaceae	1978-4494

acid:water (18:1:1) and B = acetic acid:water (2:98) with A=25% at t=0 min; A=100% at t=20 min; A=100% at t=25 min; A=25% at t=26 min (return to initial conditions). In standard analyses, 20 μl of extract were injected.

Bioactivity Guided Fractionation of Sclerochiton harveyanus Leaf Extracts The residues from the acetone extraction (1.92 g; A) or aqueous methanol extraction (2.04 g; M) from 10 g of *S. harveyanus* were dissolved in petroleum ether (50 ml) and subjected to silica gel column chromatography (Silica 60 Å,; 40 cm×2.5 cm i.d.; particle size 40–63 μm, Fisher Scientific UK) with stepwise elution first with petroleum ether:ethyl acetate (1:0, 2:1, 1:2, 0:1; 50 ml each) followed by ethyl acetate:methanol (2:1, 1:2; 50 ml each) and methanol (200 ml). The first 50 ml were discarded, and the remaining eluate was collected as 20 ml fractions from the original acetone extract, A.1–A.25, or the original methanol extract, M.1–M.25, and evaporated to dryness. Each fraction was redissolved in 2 ml methanol and evaluated by analytical HPLC and insect bioassay. The fractions of interest included two from the acetone extract (A.15 and A.19) and one from the aqueous methanol extract (M.15). Chemical constituents of the latter were purified by repetitive isolation using semi-preparative HPLC (Waters system as above) with a Merck LiChrospher 100RP-18 column (250×10.0 mm i.d.; 5 μm particle size). Operating conditions were 30°C, a flow rate of 4.5 ml/min, and an elution program based on A = methanol and B = water, with A=25% at t=0 min; A=100% at t=20 min; A=100% at t=25 min; A=25% at t=26 min (return to initial conditions).

NMR spectra were acquired in CD_3OD at 30°C on either Bruker Avance 400 MHz or Varian 500 MHz instruments using standard pulse sequences and referencing the chemical shift to TMS at 0.00 ppm. Mass spectra were obtained with a Bruker Daltonics microTOF^{TM} mass spectrometer operating in positive ESI mode. LC-APCI-MS analysis was performed using a Thermo-Finnigan system consisting of 'Surveyor' autosampler, pumps and PDA connected to an 'LCQ Classic' quadrupole ion-trap mass spectrometer fitted with an APCI source. Chromatography of 10 μl injections was performed on a Phenomenex Luna C18(2) column (150 mm×4.6 mm i.d., 5 μm particle size) using a 1 ml/min linear mobile phase gradient of water:methanol:5% acetic acid in methanol, 80:0:20 to 0:80:20 in 20 min followed by a methanol wash. Concentrated ammonia was added post column at 2 μl/min and positive ion first-order APCI-MS were recorded using the source settings: vaporizer temperature 550°C; sheath and auxiliary nitrogen gas pressures of 80 and 20 psi, a needle current of 5 μA and a heated capillary temperature of 150°C. Source induced dissociation (sidMS) was achieved by applying a collision energy of 45% in the octapole ion optics (Grayer et al., 2000).

Leaf Extract Bioactivity To evaluate toxicity of leaf extracts to *F. occidentalis*, aliquots (0.5 ml) of the acetone or 80% methanol extracts, at concentrations of 1, 10, 100, 1000 ppm, were distributed around small glass vials (2 ml) with lids in position for 2 min (N=10 for each concentration). The lids were removed and excess extract poured off, and the vials then were air dried for 20 min, thus providing a uniform coating (residue) of extract on the internal surface. Five first instars of *F. occidentalis* were placed in each vial, and mortality was recorded after 2 hr. Controls for each species of plant consisted of two sets of 10 vials rinsed with either acetone or 80% methanol.

Statistical Analysis The Shapiro-Wilk test (Field, 2005) demonstrated that the majority of the data were not from a normally distributed population, and thus non-parametric analyses were used. The differences between all controls were examined using the Kruskal-Wallis analysis of variance test (*KW*). The Mann-Whitney U test (*MW*) was carried out on each of the concentration datasets for each plant species, adjusting the significance level depending on the number of comparisons each time (Bonferroni correction). To determine whether mortality levels differed between species at each concentration the data sets were initially analyzed with the *KW* test, followed by *MW* on each of the concentration data sets to determine where the differences lay, again employing the Bonferroni correction. Differences between mortality levels achieved on exposure to acetone and 80% methanol extracts of each treatment were also examined using *MW* with the Bonferroni correction.

Activity Tests with Fractions and Isolated Compounds Fractions were tested for activity against *F. occidentalis* at concentrations of 1 to 10 ppm. Thin sections of the bean pods of *P. vulgaris* were dipped in the test solutions for several sec, in addition to rinsing the 2 ml glass vials and lids as before. Vials and cut bean sections were left to air dry for 30 min. Five first instars *F. occidentalis* were introduced into each vial (N=15) and allowed to feed on bean sections for 2 hr before assessing mortality. Control treatment vials (N=15) and cut bean sections were rinsed with acetone only. The mortality levels of thrips exposed to each of the test solutions were compared with controls, and differences between them were determined using *KW* and *MW* with the Bonferroni correction. Iridoids **1**–**4** isolated from active fractions from extracts of *S. harveyanus* were tested at a concentration of 100 ppm (N=15). The most active of the iridoids obtained from *S. harveyanus* was tested at concentrations ranging from 1 to 1000 ppm.

Choice Experiments Heliothrips haemorrhoidalis pupae were collected from the host *H. scandens* growing in the Temperate House, Kew. Uninfested leaves of *H. scandens*

were collected, rinsed in distilled water, dried, and cut into sections (2×2 cm) that were dipped in either 100 ppm acetone leaf extract of *S. harveyanus* (treatment 1; T1), 100 ppm iridoid **1** (treatment 2; T2), or acetone only (control). Three leaf discs; one from each treatment and a control, were equally spaced at the sides of a Petri-dish (9 cm) on top of filter paper dampened with distilled water. Five thrips pupae were placed in the center of the Petri dish, the lid was sealed with parafilm (N=15). The locations of the thrips were recorded after 24 and 144 hr (18L:6D) and the distribution of thrips between the treated leaf-discs (T1, T2), acetone only disc (control), and filter paper arena (center) were examined using *KW*. All pupae had eclosed prior to the 24 hr assessment. Dead thrips were not included in the final analysis.

Results

Leaf Extract Activity Test The toxicities of acetone and 80% methanol leaf extracts of the six plant species listed in Table 1 were evaluated for *F. occidentalis* (Fig. 1). Mortality varied among plant species at concentrations of 100 ppm (*KW* $P<0.05$) and 1000 ppm (*KW* $P<0.001$) (acetone extracts), and 10 ppm (*KW* $P<0.01$) and 1000 ppm (*KW* $P<0.05$) (80% methanol extracts). The acetone extract of *S. harveyanus* was more toxic than extracts of the other species at 1000 ppm (*MW*, $P<0.001$ in each case). Dose-relationships generated using Probit analysis were used to determine the bioactivity of dried *S. harveyanus* acetone extracts (LD_{50}=154 ppm). Extracts of *Plectranthus aff. barbatus* (acetone: *MW*, $P<0.005$) and *Plectranthus strigosus* (methanol: *MW*, $P<0.01$) increased the mortality of *F. occidentalis* at concentrations as low as 1 ppm when compared with controls. Overall there was little difference between the activity of acetone and methanol extracts, although in some cases the acetone extracts were more toxic (*KW*, *S. harveyanus*, $P<0.005$; *Plectranthus zuluensis*, $P<0.05$; *P. aff. barbatus*, $P<0.05$). However, only the methanol extracts of *Allagopappus dichotomus*, *Gardenia posoquerioides* and *P. strigosus* were active (Fig. 1). In contrast, the activity of *P. zuluensis* resided in the acetone extracts.

Chemical Composition of Whole Leaf Extracts of Sclerochiton harveyanus Column chromatography of acetone and aqueous methanol leaf extracts of *S. harveyanus* followed by semi-preparative HPLC of the most active fractions afforded four iridoids (Fig. 2). Fraction A.19 (14.2 mg) yielded **1** (3.8 mg, t_R 12.2 min); A.15 (44.6 mg) yielded **2** (7.0 mg, t_R 13.5 min) and **3** (2.0 mg, t_R 16.7 min). Fraction M.15 (69.2 mg) afforded **1** (1.4 mg, t_R 12.2 min) and **4** (3.8 mg; t_R 16.2 min).

Compound **1**, a white amorphous solid, had a molecular formula of $C_{24}H_{32}O_{11}$ by HRESIMS. Analysis of 1D (^{1}H, ^{13}C) and 2D NMR (COSY, HSQC, HMBC) datasets indicated that it was an ester of the iridoid, 8-epiloganic acid (HMBC connectivities are given in Table S1, Supplementary Material). The ^{1}H and ^{13}C resonance assignments for the iridoid moiety of **1** showed an excellent match to those of 8-epiloganin (the methyl ester of 8-epiloganic acid) recorded in CD_3OD (Junior, 1985). The ester group in **1** was readily identified as a 4-hydroxyphenylethyl moiety from NMR and MS data. Thus, in the ^{1}H NMR spectrum, characteristic resonances were observed at δ 4.25 (2H, td, J=6.8, 0.8 Hz, CH_2-8′), 2.85 (2H, t, J=6.8 Hz, CH_2-7′), 7.05 (2H, d, J= 8.6 Hz, H-2′/6′) and 6.72 (2H, d, J=8.6 Hz, H-3′/5′). The site of esterification was determined from a correlation in the HMBC spectrum between the 2H multiplet at δ 4.25 (CH_2-8′) and C-11 at δ 168.9. In LC-APCI-MS analyses conducted using a high cone voltage (source fragmentation), the most abundant fragment ion, at *m/z* 121, corresponded to protonated (4-hydroxyphenyl)ethylene. Compound **1**, therefore, therefore 8-epiloganic acid 4′-hydroxyphenylethyl ester (sclerochitonoside A). The ^{1}H NMR spectrum of the major component of **2** (sclerochitonoside B) was similar to that of **1**, but lacked the resonances corresponding to the glucosyl moiety. It also was noted that H-1, H-5, and H-9 of the iridoid aglycone were upfield shifted by –0.25, –0.05, and –0.34 ppm, respectively, compared to **1**. Use of correlations detected in 2D NMR experiments confirmed that sclerochitonoside B was the aglycone of sclerochitonoside A (**1**). However, the configuration at C-1 could not be determined as H-1 appeared as a broad multiplet in the ^{1}H NMR spectrum of **2**. As expected, LC-APCI-MS analysis (high cone voltage) of **2** revealed a prominent fragment ion at *m/z* 121 corresponding to protonated (4-hydroxyphenyl)ethylene. A further isolate (**3**) was obtained only in small amounts, but preliminary analysis showed that it mainly comprised an iridoid aglycone. The ^{1}H NMR spectrum of the latter was similar to that of **2**, but included an additional 3H singlet resonance at δ 3.76 typical of an aromatic methoxy group. The resonances of H-2′/6′ and H-3′/5′ were downfield shifted by +0.10 and +0.14 ppm, respectively, indicating that the site of methylation was 4′-OH. In LC-APCI-MS, the *m/z* value of 366 recorded for $[M+NH_4]^+$ of **3** was 14 amu greater than that of the corresponding ion of **2**. A prominent fragment ion was recorded at *m/z* 135, consistent with protonated (4-methoxyphenyl)ethylene. These data suggested that **3** was the 4′-methyl ether of **2**, i.e., sclerochitonoside B 4′-methyl ether. Analysis of NMR data acquired for **4** indicated that two subsets of resonances were present, corresponding to iridoid and secoiridoid moieties. The first subset was equivalent to that of 8-epiloganic acid 4′-hydroxyphenylethyl ester (**1**, sclerochitonoside A), except that in the ^{13}C NMR spectrum, C-7 was downfield shifted (+3.7 ppm), and both

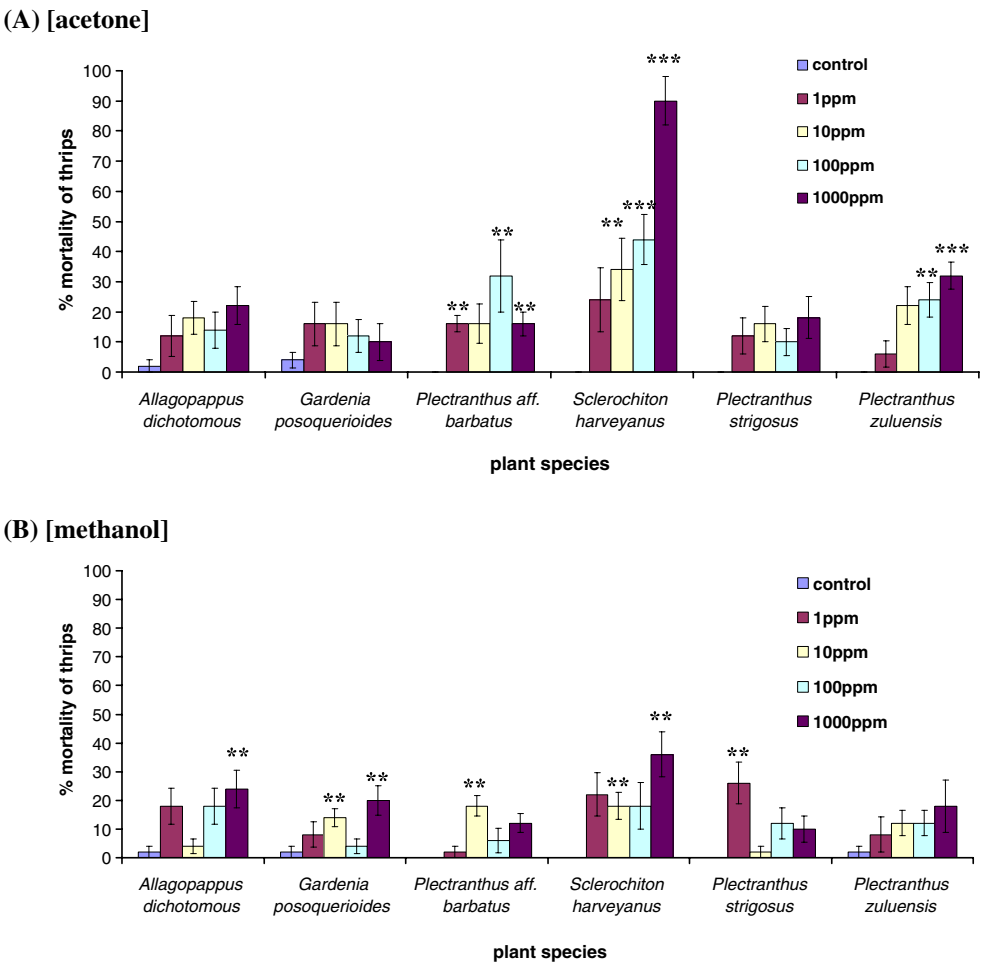


Fig. 1 Mortality of immature *Frankliniella occidentalis* (%±sem) exposed for 2 hr to materials extracted from leaves of six non-host species with acetone (**a**) or 80% methanol (**b**). Each extract was compared to the appropriate solvent control $^{**}P<0.01$, $^{***}P<0.001$, $n=10$

C-6 (– 2.2 ppm) and C-8 (–2.3 ppm) were upfield shifted. Similarly, in the ^{1}H NMR spectrum, H-7 was downfield shifted (+0.98 ppm) compared to the corresponding resonance in **1**. The second subset of resonances were those of the corresponding secoiridoid 4′-hydroxyphenylethyl ester, the structure of which was confirmed from long-range connectivities in an HMBC experiment (Table S2, Supplementary Material). Thus **4** was characterized as a dimer comprising 4′-hydroxyphenylethyl esters of iridoid and secoiridoid subunits, with 8-epiloganic acid as the parent iridoid structure, as in **1**. The linkage between the dimer subunits was defined from a correlation observed between H-7 of the iridoid moiety (subunit a) and C-7 of the secoiridoid moiety (subunit B), in an HMBC experiment. The *m/z* value of 1006 recorded for $[M+NH_4]^+$ of **4** by LC-APCI-MS was consistent with a dimeric structure. Thus, **4** was confirmed to be a dimer of sclerochitonoside A and its secoiridoid analogue, and given the trivial name, sclerochitonoside C.

8-Epiloganic acid 4′-hydroxyphenylethyl ester (sclerochitonoside A) (**1**) ^{1}H NMR (400 MHz, CD_3OD, 30°C): δ (ppm) 5.49 (1H, d, J=4.2 Hz, H-1), 7.35 (1H, d, J=1.3 Hz, H-3), 3.02 (1H, m, H-5), 2.02 (1H, ddd, J=14.0, 8.7, 4.4 Hz, CH_2-6a), 1.77 (1H, dt, J=14.0, 6.3 Hz, CH_2-6b), 3.80 (1H, ‘q’, J=5.4 Hz, H-7), 2.11 (1H, m, H-8), 2.57 (1H, m, H-9), 1.04 (3H, d, J=7.3 Hz, CH_3-10), 7.05 (2H, d, J=8.6 Hz, H-2′/6′), 6.72 (2H, d, J=8.6 Hz, H-3′/5′), 2.85 (2H, t, J=6.8 Hz, CH_2-7′), 4.25 (2H, td, J=6.8, 0.8 Hz, CH_2-8′), 4.64 (1H, d, J=7.8 Hz, H-1″), 3.18 (1H, dd, J=9.2, 7.9 Hz, H-2″), 3.36 (1H, dd, J=9.2, 8.6 Hz, H-3″), 3.24 (1H, dd, J=9.7, 8.6 Hz, H-4″), 3.30 (1H, ddd, J=9.7, 6.1, 2.2 Hz, H-5″), 3.90 (1H, dd, J=11.9, 2.2 Hz, CH_2-6″a), 3.64 (1H, dd, J=11.9, 6.2 Hz, CH_2-6″b). ^{13}C NMR (100 MHz, CD_3OD, 30°C): δ (ppm) 96.3 (C-1), 152.4 (C-3), 114.2 (C-4), 31.1 (C-5), 41.2 (C-6), 79.3 (C-7), 45.3 (C-8), 43.1 (C-9), 14.4 (C-10), 168.9 (C-11), 130.3 (C-1′), 131.0 (C-2′/6′), 116.4 (C-3′/5′), 157.1 (C-4′), 35.4 (C-7′), 66.2 (C-8′), 99.8 (C-1″), 74.8 (C-2″), 78.1 (C-3″), 71.8 (C-4″), 78.5 (C-5″), 63.0 (C-6″). LC-APCI-MS *m/z*: 514 $[M+NH_4]^+$, 335 $[((M+NH_4)–NH_3)–162]^+$, 121 $[C_8H_9O]^+$. HRESIMS *m/z*: 519.1842 $[M+Na]^+$ (calc. for $C_{24}H_{32}O_{11}Na$, 519.1837).

Sclerochitonoside B (**2**) ^{1}H NMR (400 MHz, CD_3OD, 30°C): δ (ppm) 5.24 (1H, br s, H-1), 7.36 (1H, d, J=1.1 Hz, H-3), 2.97 (1H, m, H-5), 2.01 (1H, ddd, J=13.8, 9.6, 4.0 Hz, CH_2-6a), 1.86 (1H, br m, CH_2-6b), 3.91 (1H, br m, H-7),

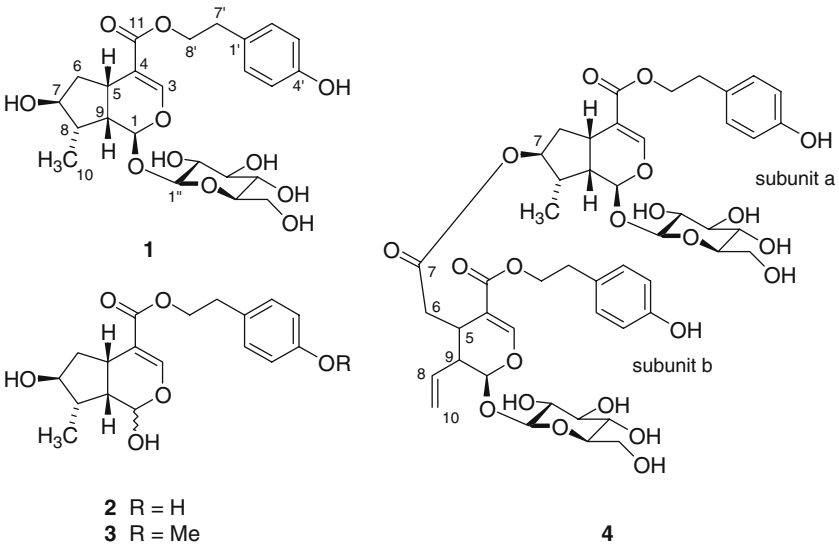


Fig. 2 Structures of four new iridoids identified in the foliage of *Sclerochiton harveyanus*: (**1**) sclerochitonoside A, (**2**) sclerochitonoside B, (**3**) sclerochitonoside B 4′-methyl ether, (**4**) sclerochitonoside C

2.12 (1H, m, H-8), 2.23 (1H, m, H-9), 1.14 (3H, d, *J*=7.1 Hz, CH_3-10), 7.05 (2H, d, *J*=8.7 Hz, H-2′/6′), 6.71 (2H, d, *J*=8.6 Hz, H-3′/5′), 2.85 (2H, t, *J*=7.0 Hz, CH_2-7′), 4.25 (2H, m, CH_2-8′). ^{13}C NMR (100 MHz, CD_3OD, 30°C): δ (ppm) 95.2 (C-1), 153.4 (C-3), 112.4 (C-4), 33.0 (C-5), 42.7 (C-6), 79.9 (C-7), 46.6 (C-8), 45.2 (C-9), 14.2 (C-10), 169.5 (C-11), 130.3 (C-1′), 131.0 (C-2′/6′), 116.3 (C-3′/5′), 157.1 (C-4′), 35.5 (C-7′), 66.2 (C-8′). LC-APCI-MS *m/z*: 352 $[M+NH_4]^+$, 121 $[C_8H_9O]^+$.

Sclerochitonoside B 4′-methyl ether (**3**) ^{1}H NMR (400 MHz, CD_3OD, 30°C): δ (ppm) 7.36 (1H, d, *J*=1.1 Hz, H-3), 2.97 (1H, m, H-5), 2.01 (1H, m, CH_2-6a), 1.88 (1H, br m, CH_2-6b), 3.89 (1H, br m, H-7), 2.13 (1H, m, H-8), 2.23 (1H, m, H-9), 1.14 (3H, d, *J*=7.1 Hz, CH_3-10), 7.15 (2H, d, *J*=8.7 Hz, H-2′/6′), 6.85 (2H, d, *J*=8.7 Hz, H-3′/5′), 2.88 (2H, t, *J*=6.9 Hz, CH_2-7′), 4.26 (2H, m, CH_2-8′) (H-1 was present as a very broad singlet at circa 5.2 ppm). LC-APCI-MS *m/z*: 366 $[M+NH_4]^+$, 135 $[C_9H_{11}O]^+$.

Sclerochitonoside C (**4**) ^{1}H NMR (400 MHz, CD_3OD, 30°C): Subunit a: δ (ppm) 5.51 (1H, d, *J*=4.6 Hz, H-1), 7.37 (1H, d, *J*=1.1 Hz, H-3), 3.02 (1H, m, H-5), 2.16 (1H, ddd, *J*=14.7, 8.7, 2.9 Hz, CH_2-6a), 1.83 (1H, m, CH_2-6b), 4.78 (1H, m, H-7), 2.34 (1H, m, H-8), 2.52 (1H, m, H-9), 1.06 (3H, d, *J*=7.4 Hz, CH_3-10), 7.04 (2H, d, *J*=8.7 Hz, H-2′/6′), 6.71 (2H, d, *J*=8.6 Hz, H-3′/5′), 2.84 (2H, t, *J*=6.9 Hz, CH_2-7′), 4.25 (2H, m, CH_2-8′), 4.67 (1H, d, *J*=7.9 Hz, H-1″), 3.21/3.20 (1H, dd, *J*=9.1, 7.9 Hz, H-2″), 3.36 (1H, m, H-3″), 3.27 (1H, m, H-4″), 3.31 (1H, m, H-5″), 3.90 (1H, dd, *J*=11.9, 2.2 Hz, CH_2-6″a), 3.64 (1H, dd, *J*=11.9, 6.3 Hz, CH_2-6″b). ^{13}C NMR (100 MHz, CD_3OD, 30°C): δ (ppm) 96.0 (C-1), 152.4 (C-3), 113.8 (C-4), 31.6 (C-5), 39.0 (C-6), 82.8 (C-7), 43.0 (C-8), 43.0 (C-9), 14.3 (C-10), 168.8 (C-11), 130.3 (C-1′), 130.8 (C-2′/6′), 116.2 (C-3′/5′), 157.1 (C-4′), 35.3 (C-7′), 66.2 (C-8′), 99.9 (C-1″), 74.6 (C-2″), 78.0 (C-3″), 71.6 (C-4″), 78.3 (C-5″), 62.8 (C-6″). Subunit b: δ (ppm) 5.47 (1H, d, *J*=4.6 Hz, H-1), 7.41 (1H, d, *J*=1.7 Hz, H-3), 3.29 (1H, m, H-5), 2.78 (1H, dd, *J*=16.3, 5.2 Hz, CH_2-6a), 2.30 (1H, dd, *J*=16.3, 8.8 Hz, CH_2-6b), 5.63 (1H, ddd, *J*=17.3, 10.3, 9.3 Hz, H-8), 2.72 (1H, m, H-9), 5.24 (1H, dd, *J*=17.2, 1.5 Hz, CH_2-10a), 5.22 (1H, dd, *J*=10.2, 1.6 Hz, CH_2-10b), 7.04 (2H, d, *J*=8.7 Hz, H-2′/6′), 6.71 (2H, d, *J*=8.6 Hz, H-3′/5′), 2.84 (2H, t, *J*=6.9 Hz, CH_2-7′), 4.25 (2H, m, CH_2-8′), 4.66 (1H, d, *J*=7.9 Hz, H-1″), 3.21/3.20 (1H, dd, *J*=9.1, 7.9 Hz, H-2″), 3.36 (1H, m, H-3″), 3.27 (1H, m, H-4″), 3.31 (1H, m, H-5″), 3.89 (1H, dd, *J*=12.0, 2.1Hz, CH_2-6″a), 3.66 (1H, dd, *J*=11.8, 5.7 Hz, CH_2-6″b). ^{13}C NMR (100 MHz, CD_3OD, 30°C): δ (ppm) 97.5 (C-1), 153.5 (C-3), 110.2 (C-4), 29.3 (C-5), 35.8 (C-6), 174.1 (C-7), 134.5 (C-8), 45.4 (C-9), 120.5 (C-10), 168.4 (C-11), 130.3 (C-1′), 130.8 (C-2′/6′), 116.2 (C-3′/5′), 157.1 (C-4′), 35.3 (C-7′), 66.2 (C-8′), 99.9 (C-1″), 74.6 (C-2″), 78.0 (C-3″), 71.6 (C-4″), 78.3 (C-5″), 62.8 (C-6″). LC-APCI-MS *m/z*: 1006 $[M+NH_4]^+$.

Sclerochitonoside A (**1**), sclerochitonoside B (**2**), sclerochitonoside B 4′-methyl ether (**3**), and sclerochitonoside C (**4**), have not been reported previously. Each compound had the common feature of esterification of the iridoid aglycone by a C-11 phenylethyl moiety (Fig. 2). Both sclerochitonoside B (**2**) and sclerochitonoside B 4′-methyl ether (**3**) lacked a glucosyl moiety at C-1. The dimer, sclerochitonoside C (**4**), comprised subunits based on the 4′-hydroxyphenylethyl esters of 8-epiloganic acid and its secoiridoid analogue. These were linked from O7 of the iridoid to C-7 of the secoiridoid. Similarly linked dimers of iridoids and their corresponding secoiridoid are uncommon, but have been reported as tricoloroside from *Loasa tricolor* (Loasaceae) (Nicoletti et al., 1991) and tricoloroside methyl ester from *Loasa acerifolia* (Müller and Weigend, 1998). In the latter examples, the iridoid subunits were loganic acid and loganin (loganic acid methyl ester), respectively.

Iridoid Activity Tests Against Thrips Exposure of *F. occidentalis* to the four iridoid compounds for 2 hr resulted in varying levels of mortality (*KW*, $P<0.05$). The two compounds which significantly increased mortality of *F. occidentalis* were sclerochitonoside A (**1**) and sclerochitonoside C (**4**) (both *MW*, $P<0.01$, applying Bonferroni correction) when compared with acetone controls ($N=15$) (Fig. 3). Increasing the concentration of sclerochitonoside A (**1**) may result in increased mortality, however, concentrations of 1 and 10 ppm were not found to result in significantly higher levels of thrips mortality when compared with the control mortality levels (Fig. 4) (*MW*, $P>0.01$ (Bonferroni correction) in both cases).

Choice experiments with *Heliothrips haemorrhoidalis* showed little evidence that these thrips were deterred by the presence of residues of crude leaf extracts of *S. harveyanus* (T1) or the iridoid sclerochitonoside A (T2) at a concentration of 100 ppm. *H. haemorrhoidalis* were equally distributed between treated and untreated leaves of *H. scandens* after 24 hr (*KW*, $P>0.05$) and 144 hr (*KW*, $P>0.05$), although after a period of 144 hr the thrips tended to be found on the control leaves rather than those treated with either the crude extract or sclerochitonoside A (Fig. 5).

Discussion

The basis of our study was to examine what effects compounds present in the leaves of 'thrips-resistant' species had on the behavior of thrips. Six species of plants located in the Temperate House, Royal Botanic Garden, Kew, which consistently remained free from thrips infestations, provided leaf extracts that demonstrated varying levels of toxicity against *F. occidentalis*. Extracts with the highest level of activity were those of *S. harveyanus*, and four novel iridoids (**1**–**4**) were isolated from these. Although iridoids were present in both acetone and methanol extracts, the most active and abundant compound, sclerochitonoside A, was more concentrated in the acetone extracts than in the aqueous methanol extracts. The relatively high activity of the acetone whole-leaf extracts of *S. harveyanus* against *F. occidentalis* may be the result of the proportionally higher concentration of sclerochitonoside A in these extracts.

Iridoid glycosides have been reported previously in the leaves, fruit, seeds, bark, and roots of many plant families including Acanthaceae (Bernays and De Luca, 1981). These compounds are frequently described as bitter tasting monoterpenoids, and have a range of activity against herbivorous insect species. Bowers (1984) recognized that iridoids were feeding and oviposition stimulants for certain specialist insects, such as the buckeye butterfly,

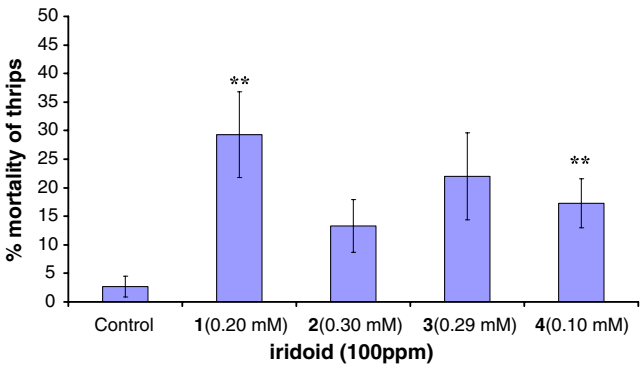


Fig. 3 Mortality of immature *Frankliniella occidentalis* (%±sem) exposed to dried residues of 100 ppm solution of iridoid isolated from leaves of *Sclerochiton harveyanus* on cut bean sections after a 2 hr period. Numbers in parentheses are mM concentrations of the compounds (**1**) sclerochitonoside A, (**2**) sclerochitonoside B, (**3**) sclerochitonoside B 4′-methyl ether, (**4**) sclerochitonoside C. Thrips mortality after exposure to iridoids was compared to the solvent control $^{**}P<0.01$, $N=15$ (**1**, **2** and **4**), $N=10$ (**3**)

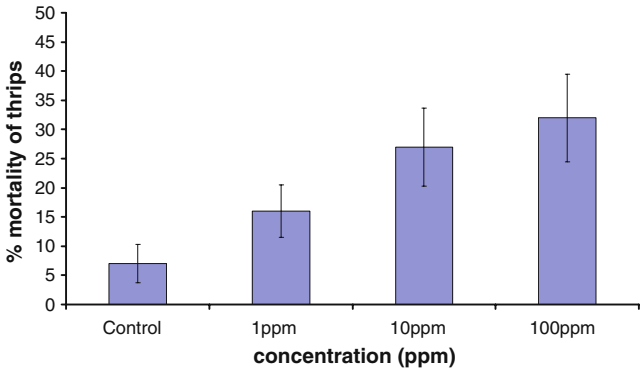


Fig. 4 Mortality of immature *Frankliniella occidentalis* (%±sem) exposed to varying concentrations of dried residues of sclerochitonoside A (**1**) (1–100 ppm) on cut bean sections after a 2 hr period. Mortality after exposure to (**1**) was compared to the solvent control $^{*}P<0.01$, $N=15$

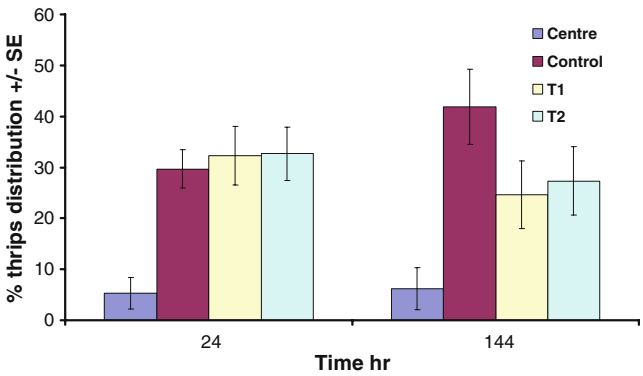


Fig. 5 Distribution of *Heliothrips haemorrhoidalis* adults (%±sem) between leaf-discs of *Hibbertia scandens* treated with 100 ppm crude leaf extract *Sclerochiton harveyanus* (T1), 100 ppm sclerochitonoside A (T2), acetone only (control) and filter paper surface (center) after 24 hr and 144 hr

Junonia coenia Hübner. Other studies have focused on the activities of iridoid glycosides against insects such as gypsy moth, *Lymantria dispar* L. and the southern armyworm, *Spodoptera eridania* (Cramer) (Bernays and De Luca, 1981; Bowers and Puttick, 1988; Fajer et al., 1992). Biere et al. (2004) concluded that the iridoid glycosides in ribwort plantain, *Plantago lanceolata* L., serve as broad-spectrum defenses against generalist insect herbivores. The iridoid-containing fractions from leaf extracts of *S. harveyanus* were highly toxic to *F. occidentalis* at relatively low concentrations, but were not conclusively deterrent to *H. haemorrhoidalis* when tested in their isolated forms.

There is little or no information on the chemistry of the foliage of the plants included in this study, with the exception of the genus *Plectranthus*, which has ethnobotanical uses (Abdel-Mogib et al., 2002; Lukhoba et al., 2006; Wellsow et al., 2006). In addition, previous studies by Scott Brown (2002) showed that cut leaves of *P. barbatus* have a deterrent effect on adult *H. haemorrhoidalis*. Phytochemical studies of *Plectranthus* have identified monoterpenoid, sesquiterpenoid, diterpenoid, and phenolic compounds (Ascensão et al., 1998; Grayer et al., 2003; Lukhoba et al., 2006; Gaspar-Marques et al., 2008). Wellsow et al. (2006) showed that diterpenoids from *Plectranthus*, including coleon A, had antifeedant activity against the generalist *Spodoptera littoralis* (Boisduval). Another diterpenoid, plectrin, isolated and characterized by Kubo et al. (1984) from leaves of *P. barbatus,* was an active antifeedant against *Schizaphis graminum* and *Pectrinophora gossypiella*. In contrast, a caffeic acid derivative, nepetoidin B, present in foliage extracts of *P. strigosus* was a phagostimulant when presented to *S. littoralis* (Grayer et al., 2003). Although the present study demonstrated that the whole-leaf extracts of the three species of *Plectranthus* are toxic against thrips, further fractionation of extracts and subsequent bioassays will be required to gain a better understanding of all the chemical constituents in these species that are responsible for the activity against *F. occidentalis* and *H. haemorrhoidalis*.

Interpreting the activity of leaf extracts of the remaining plant species included in this study against thrips is more challenging, as the phytochemistry of these plants has not been reported in detail. Novel sesquiterpene lactones and thymol derivatives have been isolated from the aerial parts of *A. dichotomus* (Gonzalez et al., 1995). Thymol has significant activity against stored-product pests (Rozman et al., 2007; Sahaf et al., 2007). Chlorogenic acid, identified in the leaf-extracts of *A. dichotomus,* is active against herbivorous insects (Bernays et al., 2000; Ikonen et al., 2001; Simmonds and Stevenson, 2001; Cipollini et al., 2008) and has been identified as a key component in chrysanthemum cultivars that are thrips-resistant (Leiss et al., 2009). Kaempferol derivatives are found in *A. dichotomus* (Gonzalez et al., 1995), and we tentatively identified a methyl ether of the flavonol, kaempferol, in the leaf extracts of *G. posoquerioides* (Scott Brown et al. unpublished data).

The leaves of the non-hosts selected for this study, with the exception of *G. posoquerioides*, are coated with sparse peltate glandular trichomes (Scott Brown and Simmonds, 2006). Such structures may provide a physical barrier against herbivorous insects and could also contain compounds that modulate thrips behavior (Wagner, 1991; Bisio et al., 1999; Roda et al., 2003). Terpenoids that have antifeedant properties occur in trichomes (Harborne, 1977; Kelsey et al., 1984). However, the lack of trichomes on the surfaces of leaves of *G. posoquerioides*, in addition to activity exclusively in the methanol extracts suggests that a different compound or compound group is key to the anti-thrips activity in this latter species. Comparing the activity levels of leaf-surface wash extracts with whole-leaf extracts could provide a valuable insight into the location of active compounds in the leaves of non-host species.

The actual role of the sclerochitonosides from *S. harveyanus* in host selection by thrips among the diverse plant collections contained within the Kew glasshouses is still unclear due to the complexity of chemically and non-chemically mediated interactions that are involved. This study indicated that crude leaf extracts of *S. harveyanus* were more toxic to *F. occidentalis* than the purified active iridoid components applied individually, thus suggesting that other components have a role in the activity against *F. occidentalis*. The presence of these compounds in the foliage of *S. harveyanus* may contribute to its observed non-host status in the glasshouses at Kew, preventing both *F. occidentalis* and *H. haemorrhoidalis* from alighting and feeding on this plant species, particularly when there are other preferred species in close proximity. Further studies are necessary in order to confirm whether the sclerochitonosides alone are responsible for the disrupted development previously observed in *H. haemorrhoidalis* when exposed to leaves of *S. harveyanus* (Scott Brown, 2002). In addition, a detailed investigation into mechanisms of the active iridoids against *F. occidentalis* may contribute towards the knowledge and subsequent development of successful control techniques against pest thrips species of high economic importance.

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The Influence of Condensed Tannin Structure on Rate of Microbial Mineralization and Reactivity to Chemical Assays

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Abstract We examined how tannin structure influences reactivity in tannin assays and carbon and nitrogen mineralization. Condensed tannins from the foliage of ten tree and shrub species and from pecan shells (*Carya illinoensis*) had different proportions of: (a) epicatechin (*cis)* and catechin *(trans)* isomers, (b) procyanidin (PC) and prodelphinidin (PD) monomers, and (c) different chain lengths. The response of each tannin to several widely used tannin assays was determined. Although there was some variation in response to proanthocyanidin (butanol/HCl) and Folin Ciocalteu assays, we did not deduce any predictable relationship between tannin structure and response to either assay. There was little variation in protein precipitation among the different tannins. To assess biological activity, six of the tannins were incubated with forest humus for 22 days. We determined that, while PC-based tannins remained at least partly extractable for the duration of the incubation, tannins with a high proportion of PD subunits rapidly became unextractable from soil. There was a positive correlation between net nitrogen mineralization and *cis* chemical structure. Carbon mineralization was enhanced initially by the addition of tannins to humus, but after 22 days, a negative correlation between the proportion of *cis* subunits and respiration was determined. Overall, we were not able to demonstrate consistent effects of structure on either microbial mineralization or reactivity to chemical assays; such relationships remain elusive.

Key Words Condensed tannin · Proanthocyanidin · Tannin structure · ^{13}C NMR · Protein binding · Acid butanol assay · Folin assay · Carbon mineralization · Nitrogen mineralization

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Introduction

Tannin is an abundant polyphenolic plant biopolymer, constituting 10–25% of foliar mass for woody species (Kraus et al., 2003a). Tannins are classified as either hydrolyzable, based on phenolic esters of a central sugar, or condensed (CT), which comprise carbon-carbon linked flavan-3-ols. Gymnosperms can synthesize only CT, but angiosperm dicots can produce both CT and hydrolyzable tannins (Bate-Smith, 1977; Hernes and Hedges, 2004). Structural categorization of CT is based on the stereochemistry at the C2 and C3 bonds, with the B ring either *trans* to the C3 hydroxyl (catechin) or *cis* (epicatechin); the number of hydroxyl groups on the B ring, with two for procyanidin (PC) and three for prodelphinidin (PD); the number of monomers in the polymers (Fig. 1) (Ayres et al., 1997; Czochanska et al., 1980; Kraus et al., 2003b; Nierop et al., 2005). Other structural variations include propelargonidin units that have one hydroxyl group on the B ring, branches formed by C4→C6 linkages, and A-type linkages based on a C2-B hydroxyl ether bond.

Tannins interact with proteins and thus play a role in forest ecosystems through their impact on herbivory (Ayres et al., 1997), allelopathy (MacKenzie and DeLuca, 2006), and carbon and nitrogen cycling (Kraus et al., 2003a). Ericaceous shrubs contain tannins that negatively affect

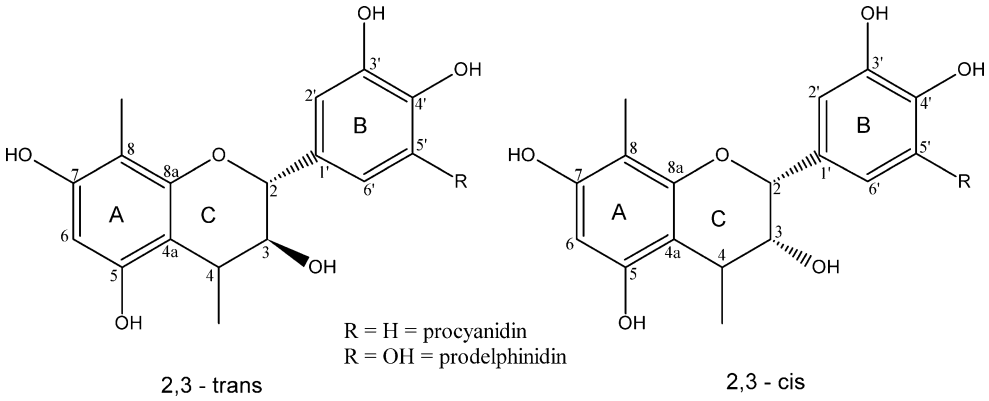


Fig. 1 Structural units of condensed tannins. Catechin type units are 2,3 *trans* (left) and epicatechin are 2,3 *cis* (right). Procyanidin (PC) subunits have two B-ring hydroxyl groups, prodelphinidin (PD) subunits have three

nutrient cycling, as demonstrated for sheep laurel (*Kalmia angustifolia* L.) in the black spruce (*Picea mariana* (Mill.) BSP) forests of Quebec and Newfoundland (Bradley et al., 2000; Joannisse et al., 2007). Wurzburger and Hendrick (2007) described similar effects of *Rhododendron maximum L.* in Appalachian hardwood forests. Negative impacts on nutrient availability also have been suggested for tannin-rich salal (*Gaultheria shallon* Pursch) (Preston, 1999) although in the redcedar (*Thuja plicata* Donn) hemlock (*Tsuga heterophylla* (Raf.) Sarg.) forests of Vancouver Island tannin-rich salal was not a primary cause of poor tree growth, but rather an indicator of poor nutrient availability, especially N limitation (Prescott and Sajedi, 2008). In addition to ericaceous shrubs, CT from other species such as *Populus* impact nutrient cycling (Schweitzer et al., 2008).

Addition of sheep laurel and balsam fir CT to humus decreased mineral nitrogen cycling (Bradley et al., 2000), but the role of CT structure was not considered. Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst) tannins added to humus from Scots pine and Norway spruce stands, or from birch (*Betula pendula* Roth) groves, decreased C respiration and increased net N mineralization (Kanerva et al., 2006; Kanerva and Smolander, 2008). Although Scots pine tannins are predominantly PD-based and Norway spruce are PC, the mineralization results could not be correlated to B-ring substitution (Kanerva and Smolander, 2008). Kraus et al. (2004) compared five purified tannins and found that tannin addition to mineral soil increased cumulative CO_2 production while net N mineralization decreased compared to the control. There was some indication that tannins with a higher proportion of PC units were less labile than those with predominantly PD units, a result supported in subsequent studies (Nierop et al., 2006a, b).

The responses of CT to different analytical methods are not consistent, therefore it has been suggested that for quantitative studies, CT standards should be purified from the species of interest (Schofield et al., 2001; Kraus et al., 2003b). However, there has been little investigation of this variability by using well-characterized purified tannins. Kraus et al. (2003b) extracted tannins from nine species and reported that increased percentage of PC units was positively correlated with the Folin-Ciocalteu (FC) and proanthocyanidin (PA) assays. Chain length had no significant effect on these assays or on protein precipitation, possibly because a relatively small range of chain lengths was investigated.

This study explores three hypotheses about the relationship between CT structure, reactivity in assays, and biological activity. The first hypothesis is that there is a structure-response relationship such that: (a) PA and FC responses increase with increasing PC (%), and (b) protein precipitation (PP) and PA responses increase with increasing CT chain length. The second hypothesis is that CT comprising high PC (%) decrease overall C mineralization. The third hypothesis is that CT containing high PD (%) decrease N mineralization. Specifically, we purified ten condensed tannins from the foliage of tree and shrub species (sheep laurel, black spruce, hemlock, redcedar, salal, balsam fir, Scots pine, jack pine (*Pinus banksiana* Lamb.), blueberry (*Vaccinnium ovalifolium)*, and Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco)] and from pecan shells (*Carya illinoensis*). The purified CT were structurally characterized using NMR, and were analyzed with three standard tannin assays (PA, FC, and PP). Ecological processes were assessed by quantifying microbial respiration and N mineralization by using humus from the forest floor of a Douglas-fir stand amended with six of the tannins.

Methods and Materials

Sample Origins Composite needle samples of jack pine and black spruce were collected from sites along the Boreal Forest Transect Case Study (BFTCS; Preston et al., 2006) in Saskatchewan and Manitoba in 2003. Oval-leaf blueberry and salal leaves were collected from research plots near Jordan River, British Columbia (BC) in 2002 and from Port McNeill, BC in 2000, respectively. Needles of western hemlock, western redcedar, and Douglas fir were collected

in Victoria, BC in 2002 and Scots pine, the only species growing in an exotic location, also was collected in Victoria in 2001. As reported previously (Preston et al., 1997), balsam fir branch tips and sheep laurel leaves were collected in 1995 in Newfoundland, and pecan tannins were extracted from bulk broken nutshells (1990 harvest) kindly supplied by Dr. S. Kays, University of Georgia, Athens, GA, USA.

Preparation and Purification of Tannins Oven dried (40°C) leaves or freeze dried needles were ground to 2 mm in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) and stored at room temperature. Tannins were extracted and purified according to the methods in Lorenz and Preston (2002) and Nierop et al. (2005). For pecan tannins, the hexane pre-extraction and solvent washing steps were omitted, as the dark red powder (packing tissue) found on the inner surfaces of the shells is almost pure condensed tannin (Preston and Sayer, 1992).

Tannin Characterization Carbon and nitrogen analysis of purified tannins was carried out by dry combustion on a LECO CNS 2000 (LECO, St. Joseph, MI, USA). Chemical structures were determined by solution-state ^{13}C nuclear magnetic resonance spectroscopy (NMR) as reported previously (Kraus et al., 2003b; Nierop et al., 2005), using 150–175 mg of tannin dissolved in 3 ml of 1:1 acetone and D_2O. Spectra were obtained at 75.47 Hz on a Bruker MSL 300 spectrometer (Bruker Instruments Inc., Karlsruhe, Germany) using a 10 mm sample tube, inverse-gated decoupling, 45° pulse, 0.5 sec acquisition time, and relaxation delays from 3.6 sec to 10 sec. In some cases, spectra were run with two delay times, and the results were averaged as no consistent differences were apparent above 3.6 sec. The free induction decays (FIDs) were processed with baseline correction and a low line-broadening of 8 Hz to optimize resolution, and chemical shifts were reported relative to tetramethylsilane at 0 ppm using the acetone peak at 30.7 ppm as a secondary standard. Three structural parameters (Fig. 1) were determined by integration of specific regions. Percentages of (a) C2-C3 *cis* vs. *trans* stereochemistry were determined by comparing the integrated areas between 75–80 and 80–85 ppm, (b) procyanidin vs. prodelphinidin B-ring structure (reported as PC%), was determined by integrating peaks at 145 and 146 ppm, and (c) average chain lengths were determined by integrating signals at 67 ppm (terminal unit) and 72 ppm (extender units). Our NMR methods are unable to detect other structural features, such as C4→C6 branching or lower levels of B-ring hydroxylation.

Tannin Assays The eleven tannins were assessed with the FC method for total phenolics, the PA method for proanthocyanidin, and the radial diffusion PP assay. Results were reported as response curves for FC and PA assays by plotting the absorbance as a function of mass of tannin. A response curve also was generated for PP by plotting the diameter of the precipitation ring against the mass of tannin.

For the FC assay (Scalbert, 1992), purified tannin was dissolved in methanol at 0.25 mg ml^{-1}, and aliquots of the solution were dried in 15 ml screw-cap vials using forced air at room temperature to yield between 0.03–0.25 mg of tannin in each vial. Samples were redissolved with 1.0 ml deionized water, 0.5 ml FC reagent (Folin-Ciocalteu phenol reagent, Sigma-Aldrich Inc., St. Louis, MO, USA) and 2.5 ml 20% (w/v) sodium carbonate. Samples were mixed and decanted into disposable cuvettes for analysis at 750 nm on a Milton Roy Spectronic 1001 Plus (Ivyland, PA, USA).

For the PA assay, samples were dispensed and dried in the vials as for the FC assay, and 5 ml of color reagent were added (5% HCl, 0.75 mg ml^{-1} $FeSO_4$, 1.3% H_2O in butanol) (Lorenz et al., 2000). Samples were vortexed prior to, in the middle of, and subsequent to heating for 1 h in a 95°C water bath. Solutions were scanned on the Milton Roy spectrophotometer between 650 nm–450 nm, and absorbances were recorded at the λ_{max} (555±2 nm). The radial diffusion assay was carried out by dispensing 20–80 μl of 12.5 mg ml^{-1} tannin dissolved in 50% aqueous methanol into wells in agarose plates containing 0.050% gelatin type B (Difco Bacto Agar Difco Laboratories, Detroit, MI, USA; Sigma Chemical Co., St. Louis, MO, USA) (Kraus et al., 2003b). The diameters of the resulting rings were measured after the agar plates were inverted and left for 24 h at room temperature.

Tannin Incubation with Humus The top two layers, L and F, of the forest floor were removed, and 300 g of the humus layer were collected from each of three locations in a Douglas fir forest in the Shawnigan Lake Research Forest near Victoria, BC in September 2003. Locations were chosen to avoid salal plants, but otherwise were randomly selected. Samples were bulked, sieved (5 mm), mixed, stored 5 days at 4°C, and conditioned at room temperature for 5 days. The humus had a moisture content of 50%, pH of 5.3 in H_2O, and organic C and N concentrations of 365 and 10.6 mg g^{-1}, respectively, determined by dry combustion (LECO CNS 2000).

Purified tannins from black spruce, balsam fir, western redcedar, jack pine, sheep laurel, or blueberry were used in the incubation. Samples were prepared in 15 ml vials by mixing 1.000 g of fresh humus with 15 mg of tannin (9 mg C g^{-1} soil). Samples were vortexed, capped loosely to maintain aerobic conditions, and incubated at 25°C in the dark.

Respiration was measured in an incremental fashion, with the amount of CO_2 produced between each of 12 time

points (4, 22, 46, 70, 94, 146, 190, 238, 291, 331, 407, and 522 h after the addition of tannin) determined in triplicate. Each replicate comprised four (t=4 or 22 h) or three vials (all other times) that were placed in a larger container at the beginning of the time increment. The container was sealed, and left to incubate at 25°C until the next sampling time, when a syringe was used to collect an aliquot of gas from the headspace of the sealed container through a septum (Bradley et al., 2000). At that time, the next set of vials was placed in a large container to measure CO_2 production during the next increment. The headspace samples were analyzed by gas chromatography with a flame ionization detector (5890 HP Gas Chromatograph, Agilent Technologies, Santa Clara, CA, USA). Carbon dioxide production (mg CO_2 g^{-1} dry soil) was calculated using a standard curve ranging from 0.1 to 5% CO_2.

Ammonium (NH_4-N) and nitrate (NO_3-N) were determined for three vials from each treatment on d 0 and d 22. Samples were extracted with 8.00 ml of 1.00 M KCl, vacuum filtered through pre-washed Whatman #2 filter paper, and frozen for subsequent colorimetric determination of NO_3-N and NH_4-N (Joannisse et al., 2007). Net N mineralization was calculated by subtracting the control value on d 0 (0.11 mg NO_3-N kg^{-1} dry soil and 2.70 mg NH_4-N kg^{-1} dry soil) from mineral N concentration on d 22.

Extractable and residual tannins were determined for triplicate vials from each treatment on d 0, 1, 2, 3, 4, 8, 11, 14, and 22. Each vial was extracted four times by shaking for 45 min with 5.00 ml of 70/30% (v/v) acetone:water and centrifuging at 1,609 x g for 10 min. The extracts were combined for PA analysis as described above. The sample residues were washed by shaking for 10 min with 5 ml methanol, followed by centrifugation, and the residues were dried in a forced-air oven at 70°C. Residues were analyzed by adding 5.00 ml of the PA reagent to each sample, heating, and centrifuging for 20 min at 1,609 x g before reading the absorbance. Concentrations of extractable and residual tannins were determined using the standard curve obtained with pure balsam fir tannins, and were corrected for the variability in response to the PA assay (Table 1). The response correction meant that for some plants, the apparent extractable tannin was >100%, but for each type of tannin the bias due to response correction should be consistent for all measurements, so the trends in tannin extractability can be established from apparent recovery.

Statistical Analysis Responses in the FC, PA, and PP assays, and C and N mineralization, were compared for differences among the different purified tannins. The data were normally distributed and were analyzed using a one-way analysis of variance (ANOVA) with a general linear model procedure followed by a least squares means multiple range test. A forward step-wise regression was used to determine relationships between chemical structure and chemical response or incubation results. All analyses were done using SAS 9.0 (SAS Institute Inc., Cary, NC, USA).

Results

Chemical Structures and Responses of Purified Tannins The ^{13}C NMR spectra were typical of condensed tannins. The 2,3 stereochemistry of the extender units for most of the tannins was dominated by *cis* configuration typical of epicatechin, but tannins from redcedar were dominated by 2,3-*trans* stereochemistry suggesting catechin-based subunits (Table 1). The extent of B-ring hydroxylation varied widely, with lowest PC% for balsam fir, hemlock, jack pine, and pecan, and highest for black spruce and blueberry. Chain lengths ranged from 2.3 to 6.3

Table 1 Chemical structure of eleven purified tannins and slope (mg^{-1}) response to proanthocyanidin (PA), folin ciocalteau (FC), and protein precipitation (PP)

Tannin	Chemical structure			Chemical response					
	Chain length	PC (%)	*cis* (%)	PA[a]		FC[a]		PP[a]	
Balsam fir	4.5	21	72	5.1	b	11.0	b	7.4	a
Black spruce	5.4	86	76	6.9	cd	14.1	cd	9.1	bcd
Blueberry	3.7	94	94	7.1	d	9.3	b	9.0	bcd
Douglas-fir	4.1	63	77	6.3	cd	11.1	b	9.8	bcde
Hemlock	5.9	22	76	5.9	b	14.6	d	10.2	def
Jack pine	5.0	23	71	6.5	cd	14.2	d	9.0	bcd
Redcedar	3.8	27	38	6.2	cd	16.2	d	10.5	ef
Sheep laurel	2.2	68	75	4.1	a	11.1	b	10.1	cdef
Pecan	5.5	24	50	3.7	a	5.8	a	7.8	a
Salal	4.6	28	75	6.0	bc	15.3	d	9.8	bcde
Scots pine	6.3	49	77	6.6	cd	12.3	bc	11.0	f

[a] Different letters indicate statistical differences at $P<0.05$

Table 2 CO_2 respiration by humus incubated for four hours with tannin (n=3), values are mean and standard error (S.E.)

Tannin	CO_2-C[a]		
	(mg g^{-1} soil hr^{-1})	S.E.	
Control	0.020	(0.002)	a
Balsam fir	0.030	(0.001)	ab
Black spruce	0.033	(0.002)	bc
Blueberry	0.025	(0.001)	ab
Jack pine	0.048	(0.008)	d
Redcedar	0.043	(0.004)	cd
Sheep laurel	0.035	(0.001)	bc

[a] Different letters indicate statistical differences at $P<0.05$

but were lower for sheep laurel. Tannins contained small amounts of N (2 to 5 mg g^{-1}) (data not shown).

Results for the response of the tannins to the chemical assays are reported as the slope of the linear response ($r^2>0.90$) (Table 1). There was at most a two-fold difference in response for the 11 different CT types in the PA assay (Table 1), and a three-fold variation in response to the FC assay (Table 1). In contrast, there was little variation in response to the PP assay (Table 1). No significant relationships were observed between any of the chemical assays and the chemical structure (*cis*%, PC%, and chain length) of the tannins ($P>0.05$).

Tannin Incubation with Humus Six purified tannins were chosen based on their variation in chemical structure (*cis*%, PC%, and chain length) for addition to forest floor humus in a 22-day laboratory incubation. Early in the incubation, addition of four of the tannins (spruce, sheep laurel, redcedar, and pine) resulted in significantly higher respiration rates relative to the control (Table 2). After 22 days, the samples amended with sheep laurel tannins had respired significantly more C (5.66 mg g^{-1}) than the control, with 5.7% net mineralization of the added tannin C (Table 3). Addition of jack pine or redcedar did not change soil microbial respiration over the 22-day trial. Addition of balsam fir, black spruce, or blueberry tannins inhibited respiration (Table 2), with blueberry tannins most inhibitory. While chain length and proportion of PC did not affect the cumulative CO_2 production, an increased proportion of *cis* subunits significantly decreased total respiration (P=0.027).

Gross inorganic nitrogen mineralization was significantly higher for humus amended with blueberry or balsam fir tannins compared to the control (Table 3). The increase in N mineralization for balsam fir tannins was due to more ammonium N formation, while for blueberry both ammonium N and nitrate N increased compared to control (Table 3). Net N nitrification was significantly higher for sheep laurel and blueberry treatments compared to the control (Fig. 2). Conversely, there were no significant differences between the control and the tannin treatments for net N ammonification (Fig. 2). As with C mineralization, PC%, and chain length were not significant factors in N mineralization, while the proportion of *cis* subunits was positively correlated with net ammonium (P=0.028) and nitrate (P=0.005) formation.

Quantitation of extractable tannins from humus is influenced by degradation and sorption of tannin. Extractable tannins were not detected in the control samples, but we measured 0.64 mg g^{-1} non-extractable tannins in the control humus. For the amended humus, extractable tannins on d 0 ranged from 34% (sheep laurel) to 121% (black spruce) of the tannin added. In all of the treatments, there was an initial rapid decrease in the amount of extractable tannins, followed by a steady decrease for the remainder of the incubation (Fig. 3). Humus treated with black spruce or blueberry tannins had the highest recoveries of extractable tannins throughout the incubation, with recoveries statistically higher than for the other four treatments on d 22 (Fig. 3). The lowest recovery of tannin initially was from

Table 3 Tannin incubation results for CO_2 cumulative respiration, gross ammonium and nitrate measurements, and total gross inorganic nitrogen (TGIN) after 22 days (mean and standard error (S.E.))

Tannin type	Cumulative CO2-C[a]			NO3-N[a]			NH4-N[a]			TGIN[a]		
	(mg g^{-1} soil)	S.E.		(mg kg^{-1} soil)	S.E.		(mg kg^{-1} soil)	S.E.		(mg kg^{-1} soil)	S.E.	
Control	5.16	(0.17)	b	2.10	(0.38)	bc	21.78	(3.13)	c	23.88	(3.49)	c
Balsam fir	4.62	(0.08)	c	2.55	(0.44)	bc	33.83	(4.64)	b	36.38	(4.84)	b
Black spruce	4.71	(0.16)	c	1.40	(0.20)	c	17.97	(0.80)	c	19.37	(0.96)	c
Blueberry	3.84	(0.20)	d	8.13	(0.55)	a	55.02	(4.58)	a	63.14	(5.00)	a
Jack pine	5.11	(0.07)	b	1.58	(0.55)	c	19.11	(2.46)	c	20.70	(2.18)	c
Redcedar	5.15	(0.10)	b	2.28	(0.36)	bc	22.32	(1.99)	c	24.61	(2.30)	c
Sheep laurel	5.66	(0.08)	a	3.47	(0.55)	b	26.42	(1.06)	bc	29.89	(1.60)	bc

[a] Different letters indicate statistical differences at $P<0.05$

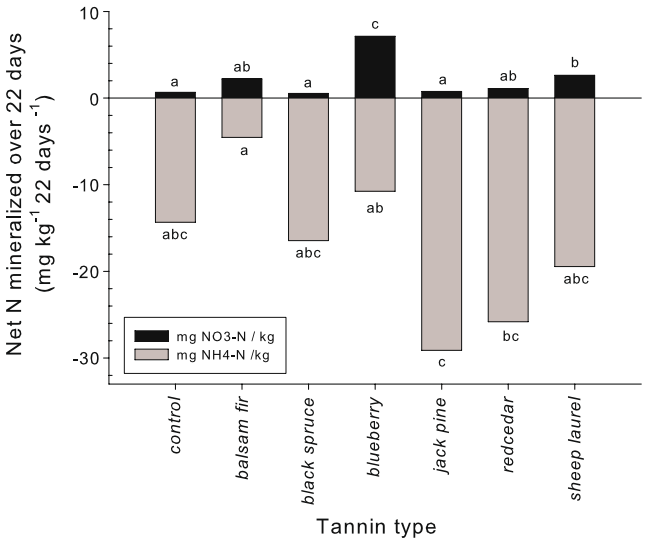


Fig. 2 Net N mineralization during the 22-d incubation of humus with tannin from balsam fir, black spruce, blueberry, jack pine, redcedar, or sheep laurel. Different letters indicate significant differences in net N mineralization between the treatments ($P<0.05$)

the sheep laurel tannins treatment, but after 5 days, and for the remainder of the incubation experiment, the lowest recoveries were from the balsam fir treatment. For the amended humus, residual tannin recovery was always less than 2% of the tannin added.

Discussion

Eleven purified condensed tannins were characterized by establishing structural details, responses to chemical assays,

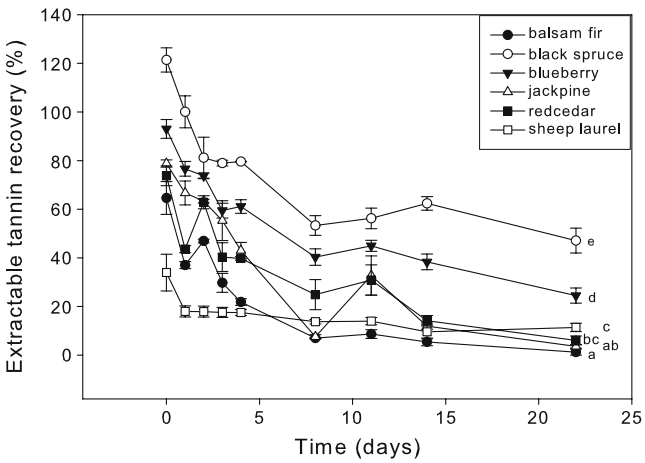


Fig. 3 Percent recovery of added tannin from balsam fir, black spruce, blueberry, jack pine, redcedar, or sheep laurel from forest humus as a function of time. The PA assay was used to determine extractable tannins. Error bars indicate one standard error ($N=3$) and different letters indicate statistical differences between tannin treatments on d 22 ($P<0.05$)

and effects on soil microbial mineralization of C and N. The ^{13}C NMR spectra were similar to those reported in other studies (Czochanska et al., 1980; Ayres et al., 1997; Kraus et al., 2003b), thus confirming that the 11 CTs were essentially free of carboxyl C and extraneous low-molecular-weight contaminants. Structural parameters found in this study (Table 1) are comparable with results from other studies, but some of our NMR data differ slightly from previously published values due to improvements in spectral processing or acquisition of new spectra. The range of values for *cis* configuration, PC%, and chain length is similar to that reported previously for boreal (Ayres et al., 1997) and temperate (Kraus et al., 2003b) forest species. Our structural parameters determined by NMR are generally supported by the characterizations that use techniques such as thermally-assisted hydrolysis and methylation-gas chromatography/mass spectrometry (THM/MS) (Nierop et al., 2005), high-performance liquid chromatography with mass spectrometry (RP- and NP-HPLC/MS) (Kanerva et al., 2006), and direct acid depolymerization (Hernes and Hedges, 2004). However, the NMR determination of chain length may not correctly represent sheep laurel tannins due to incorporation of *p*-coumaric acid units in the polymer (Nierop et al., 2005).

Our set of eleven CTs provided a wide range of chemical structures for evaluating the relationship between chemical structure and chemical reactivity. Blueberry, with the highest proportion of *cis* subunits and PC stereochemistry, had the strongest response to the PA assay, although we found no significant relationship between PA response and the chemical structural parameters that we measured, in agreement with Porter et al. (1986). This was in contrast to Kraus et al. (2003b) who found that tannins with higher percentages of *cis* subunits and PC configurations gave a stronger responses to both the FC and PA analysis. Neither *cis*%, PC%, nor chain length had an effect on PP, confirming previous results (Porter and Woodruffe, 1984; Kawamoto et al., 1990; Kraus et al., 2003b). While we found no relationship between chemical structure and chemical response, the analyses did confirm that different tannins exhibit different responses, leading us to recommend that tannin concentrations in unknown samples be accurately established by using tannin standards that are identical to those found in the sample. A better understanding of the structural factors that affect tannin response to these assays is still required.

Part of the problem in developing better structure-function relationships is that the tannin preparations typically used in this and similar studies are not single pure compounds. Our derived structural parameters were averages, as evidenced by non-integer chain lengths, and not all features were resolved. Plant tannins generally can be purified from extraneous compounds, such as sugars,

lipids, and monomers such as catechin, but may be mixtures of CT and hydrolyzable tannins (e.g., Kraus et al., 2003b), or may contain small amounts of structural units not detected by the basic NMR techniques currently in use, such as C4→C6 branching or monohydroxylation on the B ring. The latter is unlikely to make a significant contribution to structures of CT for temperate shrub and forest species (Hernes and Hedges, 2004), but the contribution of branching units (Newman et al., 1987) should be further investigated. To further elucidate the purified tannin structures other techniques could be used; e.g., THM/MS (Nierop et al., 2005), RP- and NP-HPLC/MS (Kanerva et al., 2006), or matrix-assisted laser deionication-time of flight mass spectrometry (MALDI-TOF/MS) (Li et al., 2010). Additional development of efficient purification, fractionation, or synthetic routes to more homogeneous preparations would be helpful. On the other hand, the rather disperse and heterogeneous tannins that have been widely reported are representative of what plants produce, and therefore are appropriate for ecological studies.

The ecological impact of these heterogeneous tannins can be assessed by monitoring biological activity either in the field, in microcosms, or in laboratory incubations. This study used a laboratory incubation of forest floor humus with six selected CT. After 22 days, there was no significant effect of PC hydroxylation pattern on CO_2 respiration, therefore, our hypothesis that structures with a high proportion of PC would decrease overall C mineralization was not supported. However, the study revealed a negative effect of *cis* stereochemistry on cumulative respiration. The large negative influence of blueberry tannins on C respiration may be due to its high proportion of both *cis* stereochemistry and PC substitution patterns. The results also are generally in keeping with other studies of tannin addition to humus where microbial respiration may be stimulated in the short term but is usually suppressed after 3–4 weeks (Kanerva et al., 2006; Nierop et al., 2006a,b; Nierop and Verstraten, 2006). In contrast, addition of tannins to mineral soil is reported to cause an increase or no change in respiration (Kraus et al., 2004; Madritch et al., 2007; Talbot and Finzi, 2008).

Addition of blueberry CT (94% *cis* and 94% PC) to forest humus resulted in the largest production of ammonium and nitrate in the incubation. While *cis* stereochemistry was positively correlated to net ammonium N and net nitrate N concentration, neither proportions of PC nor chain length were related to N mineralization. In general, we observed little change in N mineralization with the addition of purified tannins to humus (Table 3). Our third hypothesis, that N mineralization would be suppressed with increasing proportions of PD units, was not supported. Other studies have shown a decrease in ammonium N with the addition of tannins to humus (Bradley et al., 2000; Nierop et al., 2006a). Microbial community function is negatively impacted by the addition of tannins (Joannisse et al., 2007; Adamczyk et al., 2009), and Bradley et al. (2000) noted a decrease in microbial biomass when tannins were added to humus. Inhibited or diminished microbial communities may exhibit lower organic matter decomposition and decreased N mineralization, thus leading to the idea that tannins negatively impact nutrient cycling. While not conclusive in terms of N mineralization, our study suggests that tannin structure, particularly *cis* orientation, is important in nutrient cycling.

Analysis of tannins from forest floor is challenging due to lignin interference (Preston et al., 1997; Nierop and Filley, 2008). The few other studies to follow recovery of added condensed tannins to organic horizons were similar to ours, with rapid initial decline in extractable tannins and loss of most or all extractable tannins by 30 days (Nierop et al., 2006a, b; Kraal et al., 2009). We found that there is a negative influence of B-ring hydroxylation on extractable CT, consistent with suggestions that PD substitution may be chemically reactive with organic matter, readily forming quinones and reacting with basic amino acids (Hernes et al., 2001; Hernes and Hedges, 2004). In contrast, PC tannins may be more slowly oxidized and thus may remain extractable and detectable by PA assay. Recovery of the two high-PC tannins, blueberry and black spruce, was high compared to the rapidly declining balsam fir and sheep laurel tannins. The rapid initial loss of sheep laurel tannins may be due to short chain length and *p*-coumaric acid content, which could make it more susceptible to microbial utilization (Kanerva et al., 2006; Nierop et al., 2006a). Reports of high CT concentrations in forest floors of some black spruce stands in Canada (Lorenz et al., 2000; Preston et al., 2006) are consistent with the lower propensity of high-PC tannins to react with organic N. While forest floor tannin concentrations are affected by other factors, such as drainage and rooting, Kanerva et al. (2008) found higher forest floor CT under Norway spruce (high PC tannins) than under Scots pine (high PD tannins).

Apart from enhanced respiration of low-molecular weight fractions (phenolic monomers and tannin dimers) compared to high molecular weight tannins, specific effects of CT structure on soil processes have been difficult to demonstrate. We found that high PC tannins were more extractable than PD tannins, consistent with the greater chemical reactivity of the tri-hydroxylated B ring of PD structures (Hernes et al., 2001; Hernes and Hedges, 2004). Tannins rich in *cis* oriented subunits negatively correlated with respiration but positively related to net N mineralization. Prediction of the effect of CT structure on ecological processes was not achieved, but the results confirm that CT are a heterogeneous group that interact in different ways with ecological processes. Of interest are the long chain,

high PC (%) and high *cis* (%) structures, which seem to have major influences on C and N cycling. Further work to characterize tannin structures and properties may help to elucidate and predict their roles in ecological processes.

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1,8-Cineole Inhibits Both Proliferation and Elongation of BY-2 Cultured Tobacco Cells

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Abstract Volatile monoterpenes such as 1,8-cineole inhibit the growth of *Brassica campestris* seedlings in a dose-dependent manner, and the growth-inhibitory effects are more severe for roots than hypocotyls. The preferential inhibition of root growth may be explained if the compounds inhibit cell proliferation more severely than cell elongation because root growth requires both elongation and proliferation of the constituent cells, whereas hypocotyl growth depends exclusively on elongation of existing cells. In order to examine this possibility, BY-2 suspension-cultured tobacco (*Nicotiana tabacum*) cells were treated with 1,8-cineole, and the inhibitory effects on cell proliferation and on cell elongation were assessed quantitatively. Treatment with 1,8-cineole lowered both the mitotic index and elongation of the cells in a dose-dependent manner, and the half-maximal inhibitory concentration (IC_{50}) for cell elongation was lower than that for cell proliferation. Moreover, 1,8-cineole also inhibited starch synthesis, with IC_{50} lower than that for cell proliferation. Thus, the inhibitory effects of 1,8-cineole were not specific to cell proliferation; rather, 1,8-cineole seemed inhibitory to a variety of physiological activities when it was in direct contact with target cells. Based on these results, possible mechanisms for the mode of action of 1,8-cineole and for its preferential inhibition on root growth are discussed.

Key Words BY-2 · Cell elongation · Cell proliferation · 1,8-cineole · Monoterpene · Protoplast · Starch synthesis

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Introduction

Monoterpenes are major components of essential oils, and are wide-spread secondary metabolites in the plant kingdom. Allelopathic interactions due to monoterpenes may be common in nature (e.g., Müller, 1970). They inhibit seed germination and plant growth (Tarayre et al., 1995; Abrahim et al., 2000), causing morphological and physiological changes in plant seedlings (Einhellig and Leather, 1988; Fischer, 1991; Dudai et al., 2000). They inhibit respiration in isolated mitochondria and mitosis, deteriorate membrane integrity of treated cells, interfere with cuticular waxes, enhance transpiration, cause lipid oxidation, and disrupt microtubules (Duke and Oliva, 2004; Dayan et al., 2000; Romagni et al., 2000; Schulz et al., 2007; Zunino and Zygadlo, 2004; Chaimovitsh et al., 2010). Nonetheless, the molecular mechanism (e.g., primary target point) for the allelopathic effects of monoterpenes is still obscure.

Koitabashi et al. (1997) and Nishida et al. (2005) examined the effects of five monoterpenes produced by *S. leucophylla* (camphor, 1,8-cineole, α-pinene, β-pinene, and camphene) on germination and seedling growth of a test plant, *Brassica campestris*. The monoterpenes inhibited growth of *B. campestris* seedlings in a dose-dependent manner, and their inhibitory effects appeared to affect root

growth more severely than hypocotyl growth. The monoterpenes did not alter the sizes of matured cells in roots but effectively inhibited DNA synthesis and cell proliferation in the root apical meristem. These results suggested that monoterpenes preferentially inhibited some physiological processes involved in cell proliferation. Therefore, the observed preferential inhibition of root growth relative to hypocotyl growth might be explained by the preferential inhibition of cell proliferation, because root growth requires both cell proliferation and cell elongation whereas hypocotyl growth only requires elongation of existing cells (Obroucheva, 1999). Thus, the different sensitivities of hypocotyls and roots to monoterpenes could give us a clue towards understanding their mode of action.

In the present study, we examined the effects of a monoterpene, 1,8-cineole, on proliferation and elongation of plant cells by using BY-2 suspension-cultured tobacco (*Nicotiana tabacum*) cells as receiver cells. 1,8-Cineole was chosen because it is easy to handle (it is liquid at room temperature) and has strong inhibitory effects (Nishida et al., 2005). Tobacco BY-2 cells were chosen for the following reasons: (i) In liquid suspension culture, BY-2 cells form small cell clusters composed of several (2–16) cylindrical cells connected in tandem (Sakai et al., 2004). Since most of the surface area of individual cells is exposed directly to culture media, cells are expected to exhibit clear, synchronous, and homogeneous responses to any compound applied to the culture medium; (ii) BY-2 cells can exhibit either a high proliferation rate or drastic elongation, depending on culture conditions. Under conventional culture conditions, BY-2 cells rapidly proliferate and multiply up to 100-fold within a week (Nagata et al., 1992). In contrast, when cultured in an auxin-depleted, cytokinin-containing medium, they elongate drastically and accumulate starch without proliferation (Sakai et al., 1996). Thus, BY-2 cells should provide us with a suitable system for examining the effects of monoterpene on cell proliferation and cell elongation; (iii) A protoplast culture system, in which proliferation and elongation are induced separately depending on culture conditions (Hasezawa and Syono, 1983), is established. Because protoplasts are suspended individually without any protection from a cell wall or neighboring cells (at least initially, i.e., before regeneration of cell wall), a protoplast culture system may provide us with a more sensitive way to examine the effects of monoterpene.

We first examined the effects of 1,8-cineole on growth of tobacco seedlings to check whether the phenomena observed for *B. campestris* were also true for tobacco. Then, appropriate experimental conditions were determined by using the protoplast culture system. Finally, the effects of 1,8-cineole on cell proliferation and cell elongation were assessed quantitatively with BY-2 cell cultures. We also assessed the effects of 1,8-cineole on starch synthesis, as an example of a physiological activity other than cell proliferation and cell elongation. Based on the results, possible mechanisms for the differential inhibition of root growth and hypocotyl growth by monoterpenes as well as the mode of action of monoterpenes at cellular level are discussed.

Methods and Materials

Plant Materials, Chemicals, and Treatment of Seedlings with 1,8-Cineole Seeds of *N. tabacum* (cv. Bright Yellow-2, from which a BY-2 cell line was established) were sown on two layers of filter paper (Whatman No. 3, diam 55 mm) soaked with 3 ml of water in transparent, airtight containers (5.5 cm diam×9.6 cm height, 230 ml volume, 50 seeds per container). 1,8-Cineole, purchased from Aldrich (Milwaukee, WI, USA), was spotted onto a piece of filter paper (3×6 cm) hanging from the cap of the container and allowed to volatilize into the airspace within the container. The doses of 1,8-cineole were expressed as the theoretical concentrations in the airspace within the container, which were calculated assuming that the spotted compounds volatilize completely without adsorption or leakage. For example, spotting of 17 μl of 1,8-cineole solution (f.w.=154, d=0.921) to filter paper hanging within the 230-ml container gives final concentration of 440 μM; zero μM (control) indicates no application of 1,8-cineole liquid. (Note that actual concentrations in the airspace may be lower than those theoretical, calculated values as a result of absorption. See discussion.) The seeds/seedlings in the containers were incubated for several days at 23°C under a 14: 10 h, L:D photoperiod.

Measurement of Seedling and Cell Sizes The lengths of hypocotyls and primary roots were measured with digital calipers. Only individuals that successfully germinated were used for measurements. For determination of cell size, seedlings were fixed, washed, and cleared according to the methods of Yadegari et al. (1994). The cortex cells at the root-hair-forming region (i.e., differentiation/elongation zone in the upper part of the root) of the primary roots were observed with a microscope (BX-60, Olympus, Tokyo, Japan) equipped with Nomarski optics and their sizes measured.

Determination of Mitotic Index in the Root Apical Meristem - Root tips (5 mm long) were excised, fixed with FAA solution (formalin : acetic acid : 50% ethanol=1 : 1 : 18, v/v/v) for 12 h, rinsed with 0.1 M sodium phosphate buffer (pH 7.0), and dialyzed against 0.1 M sodium phosphate buffer containing increasing concentrations of

sucrose (10, 20, and 30%, successively). The samples were immersed finally in O.C.T. compound (Sakura Finetech, Torrance, CA, USA), quickly frozen with liquid nitrogen, and the frozen, serial sections (7 μm thick) were prepared using a cryostat (OTE Cryostat, Bright, UK) at -26°C. Sections were mounted on gelatin-coated slides and air-dried, as described previously (Tamotsu et al., 1994). They were stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) without removal of the O.C.T. compound, observed with a fluorescence microscope (BX-60, Olympus) under UV irradiation, and the percentages of the cells in a mitotic phase were counted as described in Nishida et al. (2005).

Protoplast Culture and Treatment with 1,8-Cineole Protoplasts were prepared and cultured according to the method of Hasezawa and Syono (1983) with slight modifications. BY-2 cells usually were propagated by inoculating 95 ml of fresh medium with 1.5–2 ml of suspension of stationary-phase cells at weekly intervals and incubating the culture at 26°C in the dark on a gyratory shaker (130 rpm), as described by Yasuda et al. (1988). Four-day-old BY-2 cells were converted to protoplasts by treating them with enzyme solution (0.4 M mannitol, 1% cellulase YC, 0.1% pectolyase Y23, pH 5.5) for 90 min at 30°C. The protoplasts were collected by centrifugation (260*g*, 2 min), suspended in 0.4 M mannitol solution, centrifuged again, and suspended in either 2,4-D medium (LS medium containing 3% sucrose, 0.4 M mannitol, and 0.2 mg/l 2,4-D) or BA-NAA medium (LS medium containing 3% sucrose, 0.4 M mannitol, 1.0 mg/l BA, and 0.1 mg/l NAA) at a density of $0.5–1\times10^5$ cells/ml. The protoplast suspension was dispensed into 50-ml Erlenmeyer flasks (4.6 ml per flask), and cultured at 26°C in the dark without shaking. For treatment with 1,8-cineole, the protoplast-containing flasks were settled, one-by-one, in transparent, airtight containers (5.5 cm diam×9.6 cm height, 230 ml volume). 1,8-Cineole was applied either by spotting appropriate volumes of 1,8-cineole liquid onto a piece of filter paper hanging from the cap of the container (volatilization method) or by adding it directly into the culture medium in the flask (direct-addition method). In both cases, the caps of the containers were closed immediately after the application of 1,8-cineole, and the compound was allowed to volatilize into the airspace within the container. The final concentrations of 1,8-cineole in the airspace were calculated assuming that the compound volatilized completely without adsorption or leakage, as described above.

Cell Culture and Treatment with 1,8-Cineole Seven-day-old (stationary-phase) BY-2 cells were transferred to either fresh D-medium (modified LS medium containing 0.2 mg/l 2,4-D) or fresh B-medium (modified LS medium containing 1 mg/l BA) at a 1:20 dilution, and dispensed into 50-ml Erlenmeyer flasks (9.2 ml per flask). For treatment with 1,8-cineole, the cell suspension-containing flasks were settled, one-by-one, in transparent, airtight containers described above, and were fixed to prepare for shaking-culture. 1,8-Cineole was applied via the direct-addition method only. After the addition of 1,8-cineole, the caps of the containers were closed immediately, and the cells were cultured for 2 days at 26°C in the dark on a gyratory shaker (130 rpm).

Measurements of Mitotic Index, Cell Length, and Starch Content of the Cells/Protoplasts For measurements of mitotic index, protoplasts and cells were fixed with 1% formaldehyde, stained with 1 μg/ml DAPI, and examined with a fluorescence microscope (BX-60) under UV-irradiation. For measurements of the cell (or protoplast) length, samples (with or without fixation with 1% formaldehyde) were observed with a microscope, photographed, and the length measured. The starch content of BY-2 cells was measured according to the method of Sakai et al. (1996). In short, BY-2 cells present in 1 ml of cell suspension were collected by centrifugation, converted to protoplasts by incubation for 1 h at 30°C in enzyme solution (0.4 M mannitol, 1% cellulase YC, 0.1% pectolyase Y23, pH 5.5), and solubilized by adding 1/10 vols of 10% SDS and incubation for additional 30 min at 37°C. Insoluble materials, including starch granules, were collected by centrifugation, re-suspended in 1 ml of hot 80% ethanol, and collected again by centrifugation. The starch was solubilized in hot water, extracted with perchloric acid, and quantified by the phenol-sulfuric acid method (Dubois et al., 1956) using a dilution series of glucose solution as a standard.

Estimation of Half-maximal Inhibitory Concentrations (IC_{50}) We emploied IC_{50} value as the parameter to represent the sensitivities of the biological activities of interest to 1,8-cineole. The IC_{50} values were simply estimated from semi-log graphs of dose–response curves, without any mathematical model-fitting (Belz et al., 2005).

Results

Effects of 1, 8-Cineole on the Growth of Tobacco Seedlings In the absence of 1,8-cineole, germination of tobacco seeds proceeded rapidly and synchronously under our experimental conditions. Almost 100% germination was achieved 5 days after sowing, and the length of hypocotyls and primary roots reached their maximum values by day 10 (data not shown). Thus, the effects of various concentrations of 1,8-cineole were examined on day 10.

Germination of tobacco seeds (Fig. 1, top) was largely unaffected by 1,8-cineole concentrations of 500 μM or less. The germination rate suddenly dropped to 60% at 1,000 μM, and further dropped to 12% at 2,000 μM. The concentration of 1,8-cineole required to lower the germination rate to 50% of the control value (IC_{50}) was about 1,200 μM. Hypocotyl growth (Fig. 1, middle) was not inhibited with 1,8-cineole concentrations up to 200 μM. 1,8-Cineole stimulated hypocotyl growth slightly in a dose-dependent manner within this low-concentration range (rs=0.94, P<0.05), indicating hormesis (Duke et al., 2006; Calabrese et al., 2009). At higher concentration ranges, 1,8-cineole inhibited hypocotyl growth in a dose-dependent manner. The IC_{50} for hypocotyl growth was about 720 μM. Root growth (Fig. 1, bottom) was not stimulated by any concentrations of 1,8-cineole tested; 1,8-cineole shortened root length even in the low concentration range (≤ 200 μM). The IC_{50} value for root growth was 410 μM, indicating that root growth was more sensitive to 1,8-cineole than was hypocotyl growth.

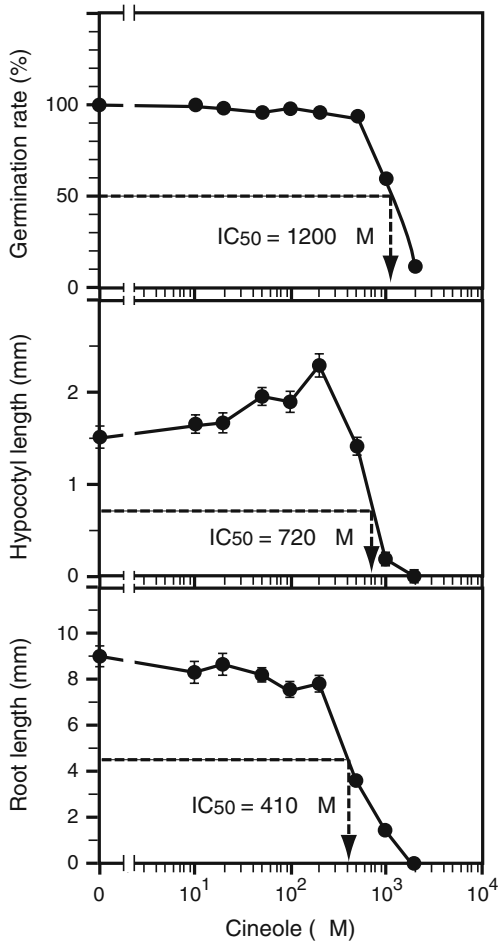


Fig. 1 Effects of various concentrations of 1,8-cineole on *Nicotiana tabacum* seed germination and seedling growth. Germination rate (*top*), hypocotyl length (*middle*), and root length (*bottom*) were measured on day 10. Each data point for germination rate was calculated from the results of all 50 individuals contained in a single container, while each for hypocotyl and root length represents the average value from 30 individuals that successfully germinated. Vertical bars represent standard errors. IC_{50} values calculated from the data are shown in respective graphs

Next, relative contribution of cell elongation and cell proliferation to the root-growth inhibition by 1,8-cineole was examined (Fig. 2). The concentration of 1,8-cineole was set at 440 μM, where hypocotyl length was almost the same as the control value, while root length was reduced by approximately 50% (Fig. 2A). Treatment with 440-μM 1,8-cineole did not reduce the size of cells in the elongation/differentiation zone of tobacco roots, but it lowered the mitotic index in the root apical meristem to less than half the control value (Fig. 2B), indicating that cell proliferation in the root apical meristem was selectively inhibited, while cell expansion in the upper region of the root was largely unaffected with this concentration.

Thus, the results obtained for tobacco seedlings (i.e., preferential inhibition of root growth over hypocotyl growth, relative insensitivity of cell elongation in the upper region of the root as compared to cell proliferation in the root apical meristem) were essentially the same as those obtained previously for *B. campestris* seedlings (Koitabashi et al., 1997; Nishida et al., 2005).

Effects of 1,8-Cineole on the Proliferation and Elongation of Protoplasts Prepared from BY-2 Cultured Tobacco Cells We examined the effects of 1,8-cineole on cell proliferation and cell elongation by using protoplasts prepared from BY-2 cultured tobacco cells. When cultured in 2,4-D medium, BY-2 protoplasts proliferated actively (with mitotic indices of about 3% from day 2 to day 6), but their sizes did not increase significantly during the culture period (Fig. 3A). In contrast, when cultured in BA-NAA medium, they ceased proliferation and elongated drastically by day 7 (Fig. 3B). Thus, BY-2 protoplasts cultured in 2,4-D medium and those cultured in BA-NAA medium were used to examine the effects of 1,8-cineole on cell proliferation and on cell elongation, respectively. In the experiments with protoplast cultures, 1,8-cineole was applied using the two different modes, i.e., the volatilization method and the direct-addition method (Fig. 4A).

Figure 4B shows the mitotic indices of BY-2 protoplasts cultured in the presence of various concentrations of 1,8-cineole in 2,4-D medium for 2 days. In either of the two application modes, relatively high concentrations of 1,8-cineole were necessary to lower the mitotic index. With the protoplast culture system, IC_{50} values of 1,8-cineole for proliferation were estimated to be about 2,000 μM for the volatilization method and 100 μM for the direct-addition method. Figure 4C shows the length of BY-2 cells cultured in the presence of various concentrations of 1,8-cineole in BA-NAA medium for 7 days. Normally, the cell wall should have regenerated by day 7. In either of the two

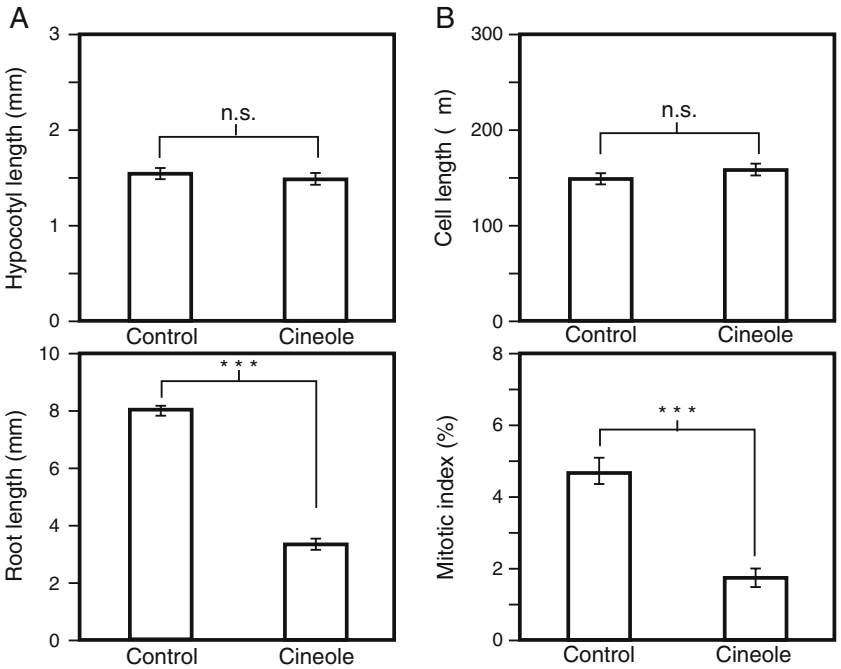


Fig. 2 Effects of 1,8-cineole on growth of *Nicotiana tabacum* seedlings. **A** Effects on hypocotyl length and root length. *N. tabacum* seedlings were grown in the absence (*control*) or presence (*cineole*) of 440 μM of 1,8-cineole for 10 days, and the hypocotyl length (*top*) and root length (*bottom*) were measured. Each datum represents the average of 20 individuals that successfully germinated. Vertical bars represent standard errors. **B** Effects on the cell elongation and cell proliferation in the affected roots. *N. tabacum* seedlings were grown in the absence (*control*) or presence (*cineole*) of 440 μM 1,8-cineole for 10 days, and the sizes of matured cells (*top*) and mitotic index in the root apical meristem (*bottom*) were measured. For cell length (*top*), each datum represents average value of 60 cells derived from more than 4 individuals. Vertical bars represent standard errors. For mitotic index, each datum represents the average value from 8 (*control*) or 7 (*cineole*) individuals. Vertical bars represent standard errors. n.s.; not significant ($P>0.05$), ***; $P<0.001$, Student's *t*-test

application modes, 1,8-cineole lowered cell length in a dose-dependent manner and completely inhibited cell elongation at the highest concentration tested (1,000 μM). IC_{50} values for cell elongation were about 140 μM for the volatilization method and 30 μM for the direct-addition method. These results demonstrate that (i) the direct addition method was more effective than the volatilization method, and (ii) in a BY-2 protoplast culture system, cell elongation was more sensitive to 1,8-cineole than cell proliferation.

Effects of 1,8-Cineole on Proliferation, Elongation, and Starch Accumulation of BY-2 Cells We examined the effects of 1,8-cineole on BY-2 cells that retained intact cell walls. BY-2 cells proliferate actively without significant increase of cell volume when cultured in D-medium that contains 0.2 mg/l 2,4-D, whereas they increase in cell volume (mostly due to cell elongation) and accumulate a large amount of starch without proliferation when cultured in B-medium that contains 1 mg/l BA (Sakai et al., 1996). Accordingly, BY-2 cells cultured in D-medium were used to examine the effects of 1,8-cineole on cell proliferation, while those cultured in B-medium were used to examine the effects on cell elongation and starch accumulation. As the differentiation of cells in the two culture media becomes clear 2 days after transfer (Sakai et al., 1996), measurements were done on d 2. Based on the results of the experiments with the protoplast culture system, only the direct-addition method was used to apply 1,8-cineole.

1,8-Cineole lowered the mitotic index in a dose-dependent manner, and the IC_{50} value for proliferation was about 140 μM (Fig. 5A). 1,8-Cineole also decreased cell length in a dose-dependent manner (Fig. 5B), and at high concentrations (120 or 240 μM), the cell size did not increase as compared to the value at the start of the culture (105 μm). The IC_{50} value for cell elongation was about 30 μM, and was lower than the IC_{50} value for cell proliferation. 1,8-Cineole also lowered starch content in a dose-dependent manner. IC_{50} value for starch accumulation was about 80 μM and was also lower than the IC_{50} value for cell proliferation. These results demonstrate that the inhibition by 1,8-cineole is not specific to cell proliferation.

Discussion

We found here that the effects of 1,8-cineole on *N. tabacum* seedlings were essentially the same as those on *B. campestris* seedlings; 1,8-Cineole preferentially inhibited

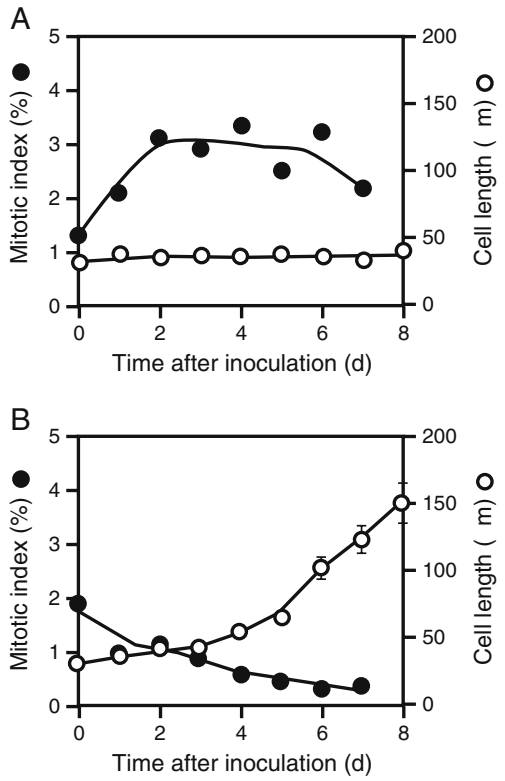


Fig. 3 Behavior of BY-2 protoplasts/cells during culture. Four-day-old BY-2 cells were converted to protoplasts and cultured in either 2,4-D medium **A** or BA-NAA medium **B**, and the changes in the mitotic index (●) and cell length (○) were followed during the culture. Each data point for mitotic index was determined by counting more than 1,000 cells/protoplasts. Each point for the length of cells/protoplasts represents the average value from 30 (for 2,4-D medium) or 60 (for BA-NAA medium) cells/protoplasts. Vertical bars represent standard errors

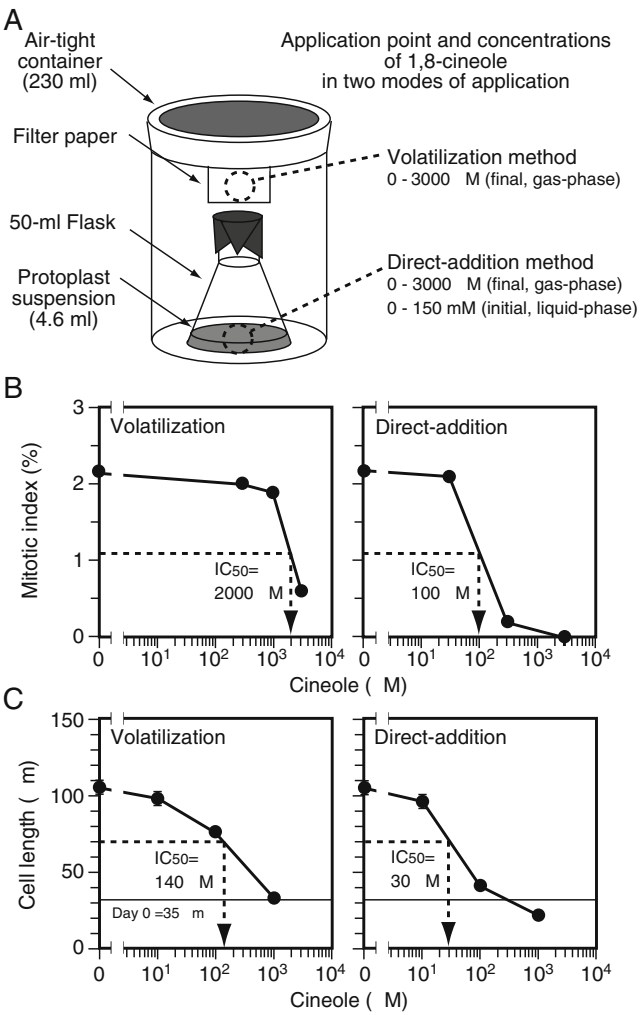


Fig. 4 Effects of 1,8-cineole and its application methods on the proliferation and elongation of BY-2 protoplasts/cells. **A** Schematic representation of the experimental system. 1,8-Cineole was applied to either filter paper hanging from the cap of the air-tight container (volatilization method) or the culture medium in the flask (direct-addition method). The calculated final concentrations of 1,8-cineole in the airspace (gas-phase) were 0 to 3,000 μM. In direct-addition method, the initial concentrations of 1,8-cineole in the culture medium (liquid-phase) were 50 times higher than the calculated final concentrations in the airspace (gas-phase). (**B**) Effects of 1,8-cineole on the proliferation of BY-2 protoplasts grown in 2,4-D medium. Various doses of 1,8-cineole were applied through either the volatilization method (*left*) or the direct-addition method (*right*), and the mitotic index was examined on d 2. Each data point was determined by counting more than 1,000 cells/protoplasts. **C** Effects of 1,8-cineole on the elongation of BY-2 protoplasts grown in BA-NAA medium. Various doses of 1,8-cineole were applied through either the volatilization method (*left*) or the direct-addition method (*right*), and cell length was examined on day 0 and day 7. Each point represents the average value from 200–600 cells/protoplasts. Vertical bars represent standard errors. In **B** and **C**, IC_{50} values estimated from the data are shown in the respective graphs

root growth over hypocotyl growth (Fig. 1). In the affected roots, cell size in the elongation zone (upper region of the root) was largely unaffected, whereas cell proliferation in the root apical region was severely inhibited (Fig. 2). These results indicate that the mode of action of growth inhibition by 1,8-cineole is common within the two plant species. Thus, we consider that tobacco, especially cultured tobacco cells, can be used as experimental material to examine the mode of action of 1,8-cineole at the cellular level.

We compared the effectiveness of two modes of application of 1,8-cineole, i.e., the volatilization method and the direct-addition method, by using a protoplast culture system (Fig. 4), and we found that the direct-addition method was more effective. This result is quite reasonable when the predicted behavior of the applied 1,8-cineole is considered. In the volatilization method, applied 1,8-cineole first volatilizes into air and then dissolves into a liquid phase. Thus, the concentrations of 1,8-cineole that the cells experience should gradually increase and finally reach the highest values determined by equilibrium between the liquid and gas phase. In the direct-addition method, the cells should experience the highest concentrations of 1,8-cineole immediately after application of the compound. Then, concentrations should decline to the lower values determined by the equilibrium. Thus, in the latter method,

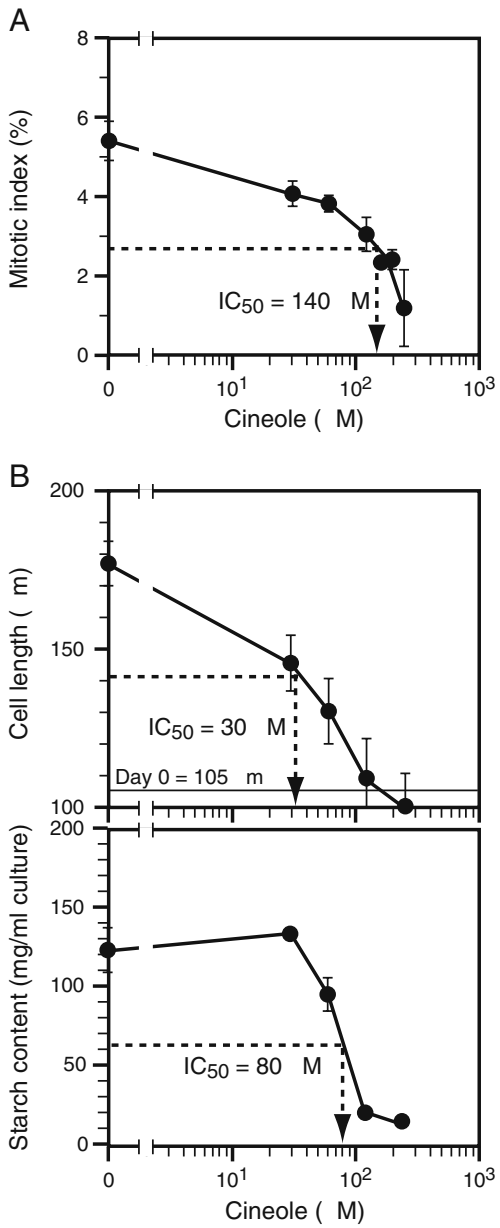


Fig. 5 Effects of 1,8-cineole on the proliferation and elongation of BY-2 cells. **A** The effects of a range of doses of 1,8-cineole on the proliferation of BY-2 cells cultured in D-medium are shown. The mitotic index was examined on d 2. The average values from three independent experiments are shown. Vertical bars represent standard errors. **B** The effects of a range of doses of 1,8-cineole on elongation (*top*) and starch accumulation (*bottom*) in BY-2 cells cultured in B-medium. Cell length and starch content were examined on d 2. The average values from three independent experiments are shown. Vertical bars represent standard errors. In **A** and **B**, 1,8-cineole was applied through the direct-addition method only. The estimated final concentrations of 1,8-cineole in the airspace (gas phase) were 0 to 240 μM, while the initial concentrations in the culture medium (liquid phase) immediately after application were 25 times higher. IC_{50} values estimated from the data are shown in respective graphs

the actual concentration that the cells experience should be higher and the time at which the cells experience the highest concentration should be earlier.

Because protoplasts lose their cell wall and are completely separated from neighboring cells, we expected that they might exhibit higher sensitivity to externally added compounds as compared to cultured cells that have intact cell walls and neighboring cells. For this reason, initially we examined the effects of 1,8-cineole on BY-2 cells using protoplasts (Figs. 3 and 4). However, IC_{50} values obtained for protoplasts and intact cells were essentially the same (compare Figs. 4 and 5), indicating that cell wall and neighboring cells did not function as a barrier to the permeation of 1,8-cineole into the cells.

In contrast to our initial expectation, 1,8-cineole inhibited cell elongation more efficiently than cell proliferation; the IC_{50} value for cell proliferation was always higher than that for cell elongation, irrespective of the application methods (Fig. 4) or the condition (i.e., presence or absence of cell wall) of receiver cells (Figs. 4 and 5). Moreover, 1,8-cineole also inhibited starch accumulation with an IC_{50} value lower than that for cell proliferation (Fig. 5). These results demonstrated clearly that the inhibitory effect of 1,8-cineole was not specific to cell proliferation; rather, 1,8-cineole appeared to affect a wide spectrum of cellular activities in an almost non-specific manner when target cells were efficiently exposed to the compound.

Despite numerous reports on inhibitory effects of monoterpenes, their mechanisms of action remain obscure. Recently, Chaimovitsh et al. (2010) reported that microtubules are an intracellular target of a monoterpene, citral. However, they also reported that the effects of citral and microtubule inhibitor oryzalin on plant seedlings are different from each other, suggesting that there should be additional target(s) of the monoterpene. Our observation that starch accumulation was highly sensitive to 1,8-cineole (Fig. 5) also suggests the presence of additional target(s), as starch accumulation itself does not seem so tightly associated with microtubule function. Then, what is the plausible target of monoterpenes?

A reduction in respiratory oxygen consumption resulting from monoterpene treatment has been reported in a number of studies (e.g., Müller et al., 1968; Peñuelas et al., 1996). Moreover, monoterpenes affect the respiratory activity of isolated mitochondria (Müller et al., 1969; Abrahim et al., 2000, 2003a) via uncoupling of oxidative phosphorylation and inhibition of electron transfer (Abrahim et al., 2003b). The lipophilic property of monoterpenes (Weidenhamer et al., 1993), as well as lipid oxidation and deterioration of membrane integrity in plant cells exposed to monoterpenes (Lorber and Müller, 1976; Fischer, 1986; Zunino and Zygadlo, 2004) suggests that biological membranes,

including mitochondrial membranes, are the primary target of monoterpenes. Among all the possible effects on biological membranes, the deleterious effects on mitochondrial membranes should cause inhibition of mitochondrial energy metabolism and result in disturbances in a wide range of physiological and biochemical processes within the cell.

Our results rule out the possibility that preferential inhibition by 1,8-cineole of root growth is explained by preferential inhibition of cell proliferation; 1,8-cineole inhibited multiple distinct biological processes (cell proliferation, cell elongation, and starch synthesis) in cultured cells, and the sensitivity of cell proliferation to 1,8-cineole was, contrary to our initial expectation, relatively low (Figs. 4 and 5). Then, in plant seedlings, why is root growth more sensitive than hypocotyl growth to 1,8-cineole?

One possibility is that the actual concentration of 1,8-cineole may be higher around roots (liquid/solid phase) than around hypocotyls (gas-phase). In nature, soil colloids adsorb volatile monoterpenes in the atomosphere and exhibit toxicity for several months (Müller and Moral, 1966). Under our experimental conditions for the seedling assay, monoterpenes within an air-tight container might absorb to the wet filter paper wad used as supporting substance, resulting in higher concentrations of the compound around the roots as compared to the concentrations around the aerial tissues. (This also suggests that the actual concentrations of 1,8-cineole in the gas-phase should be lower than the calculated values.) However, root growth was more sensitive to 1,8-cineole than hypocotyl growth even when the seedlings were grown under conditions in which both hypocotyls and roots were in equal contact with the filter paper, and therefore, the concentration of 1,8-cineole surrounding the two organs should have been equal (Nishida et al., 2005). Thus, differences in the actual concentrations around roots and hypocotyls do not seem to play a critical role under our experimental conditions.

Another possible explanation is that root surfaces may have higher permeability to 1,8-cineole as compared to hypocotyl surfaces, resulting in higher doses of the compound within root tissues as compared to those within hypocotyl tissues. The surface of aerial parts of the plant body is covered with a well-developed cuticle layer, while that of roots is not (Bessire et al., 2007). In addition, a permeability assay, based on staining with toluidine blue dye (Bessire et al., 2007), demonstrated differential permeability (and thus differential development of a cuticle layer) within a root; in various plant species, the root tip region that includes the root apical meristem exhibited higher permeability than that of the upper region of the same root, which corresponded to elongation/differentiation zone (unpublished data). Thus, differential permeability due to differential development of a cuticle layer might be involved in not only preferential inhibition of root growth over hypocotyl growth but also preferential inhibition of cell proliferation in the root apical region over cell elongation in the upper region of the same root. The relationship between development of the surface barriers, such as cuticle layer, and sensitivity to monoterpenes is now under further investigation.

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Volatile and Semivolatile Compounds in Gray Catbird Uropygial Secretions Vary with Age and Between Breeding and Wintering Grounds

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Abstract The uropygial secretions of some bird species contain volatile and semivolatile compounds that are hypothesized to serve as chemical signals. The abundance of secretion components varies with age and season, although these effects have not been investigated in many species. We used solid-phase microextraction headspace sampling and solvent extraction coupled with gas chromatography–mass spectrometry to detect and identify volatile and semivolatile chemical compounds in uropygial secretions of gray catbirds (*Dumetella carolinensis*). We identified linear and branched saturated carboxylic acids from acetic (C2) through hexacosanoic (C26); linear alcohols from decanol (C10) through docosanol (C22); one aromatic aldehyde; one monounsaturated carboxylic acid; two methyl ketones; and a C28 ester. We tested for the effect of age on signal strength and found that juvenile birds produced greater amounts of volatile C4 through C7 acids and semivolatile C20 through C26 acids, although the variation among individuals was large. Adult birds displayed small concentrations and minimal individual variation among volatile compounds, but produced significantly higher levels of long-chain linear alcohols than juvenile birds. We tested for the effects of season/location by sampling adult catbirds at their Ohio breeding grounds and at their Florida wintering grounds and found that the heaviest carboxylic acids are significantly more abundant in secretions from birds sampled during winter at the Florida site, whereas methyl ketones are more abundant in birds sampled during summer on the Ohio breeding grounds. We observed no effect of sex on semivolatile compounds, but we found a significant effect of sex on levels of carboxylic acids (C4 through C7) for juvenile birds only.

Key Words Gray catbird · *Dumetella carolinensis* · Solid-phase microextraction · SPME · Gas chromatography–mass spectrometry · GC-MS · Principal component analysis · PCA · Uropygial gland · Preen gland

Introduction

The uropygial, or preen, gland of birds produces a variety of chemical compounds with diverse functions. Among these functions are the water-proofing properties of the secretions' waxy components. In mallards, for example, blocking access to the uropygial gland for 3 months resulted in significant loss in plumage quality and increased water retention (Giraudeau et al., 2010). Volatile compounds, capable of being sensed at a distance, may serve as intraspecific chemical signals. For example, individual odorant signatures have been identified in petrels (Bonadonna et al., 2007), red junglefowl (Karlsson et al., 2010), and dark-eyed juncos (Whittaker et al., 2010). These individual signatures have been associated with mate choice and recognition (Mardon et al., 2010; Whittaker et al., 2010) and with parenting behaviors (Whittaker et al., 2009). Volatile compounds also may serve in interspecific communication or defense. For example, the positive correlation between uropygial gland size and mite abundance in passerines suggests that the gland's secretions are subject to selective pressures from mites (Galván et al., 2008). Antimicrobial compounds found in the uropygial secretions of hoopoes are produced by symbiotic bacteria in the gland (Martín-Vivaldi et al., 2010), suggesting that the origin of

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functional compounds in the uropygial secretions of other avian species may be more complex than previously appreciated.

Among the uropygial secretion profiles that have been characterized, several exhibit variation with season. In a study examining 19 sandpiper species, Reneerkens et al. (2002) found that all species switched from lower molecular weight monoesters to higher molecular weight diesters during the period of courtship and incubation. An olfactory-searching dog, used as a proxy for a nest-seeking predator, had greater difficulty sensing the heavier diester compounds than the more volatile monoesters, supporting the hypothesis that the switch to diester waxes enables olfactory crypsis at the nest (Reneerkens et al., 2005). That the diester uropygial secretion is produced by the parent(s) engaged in incubation and not by the non-incubating parent further supports the crypsis hypothesis (Reneerkens et al., 2007). Seasonal variation in volatile compounds has been demonstrated in the dark-eyed junco (Soini et al., 2007). Most notably, linear alcohols were consistently found at higher levels in captive birds maintained under long photoperiod to simulate the breeding season compared with birds reared under short photoperiod, simulating nonbreeding conditions. Such trends also were found among males in some ketones and in linear and branched carboxylic acids, although individual variation in these compounds was large. Whittaker et al. (2010) further investigated seasonally variable compounds and identified a "female-like" volatile profile containing mostly even-numbered carboxylic acids and a "male-like" profile rich in methyl ketones. They also found that secretion compounds provided information about individual identity and could distinguish between two genetically isolated populations of juncos.

Secretion profiles also vary with age. Examination of diol esters from chickens revealed significant shifts in chain length and diastereomer composition as birds matured from 3 to 13 months, after which only minor composition shifts were observed (Kolattukudy and Sawaya, 1974). Sandilands et al. (2004) sampled uropygial secretions from chickens starting at 10 weeks of age and quantified fatty acids derived from ester hydrolysis. They found a significant effect of age on the saturated fatty acids that constituted the majority of the total lipid content. Because secretion profile changed at the onset of laying, they speculated that the shift was a result of changes in sex hormone levels associated with sexual maturity. The significance of hormonal state also was demonstrated in studies of mallards, in which injection of estradiol into male and female birds in the nonmating season induced a shift from short-chain monoesters to long-chain diester compounds (Bohnet et al., 1991).

In a previous study (Whelan et al., 2010), we sampled uropygial secretions from captive gray catbirds and analyzed the chemical composition of the secretions using solid-phase microextraction (SPME) and gas-chromatography mass-spectrometry (GC-MS). We found that linear and branched carboxylic acids were the predominant volatile compounds in the secretions, and that their signal strengths varied significantly with exogenous testosterone and with photoperiod but that sex had no significant effect. As a result of the experimental design, that study focused exclusively on the most volatile components of the secretion samples, those containing up to six carbon atoms, whereas in other bird species uropygial secretions containing linear alcohols and carboxylic acids with much longer aliphatic chains have been identified (Sandilands et al., 2004; Montalti et al., 2005; Bonadonna et al., 2007; Soini et al., 2007; Karlsson et al., 2010; Zhang et al., 2010).

In this study we used a combination of analytical methods (SPME/GC-MS and solvent extraction/GC-MS) to obtain a more complete profile of the volatile and semi-volatile chemical components of gray catbird uropygial secretions. We then tested whether the abundance of these compounds varied with phase of life history in free-ranging birds. Specifically, we examined the effect of age by comparing secretions of adult birds to those of juvenile birds, thus testing the hypothesis that if secretions have important functions, juvenile birds may allocate fewer resources to their production given the competing energetic demands of rapid growth. We also examined the effect of breeding by comparing adult birds captured during the breeding season in Ohio to adult birds captured during the non-breeding season in the winter in Florida. In these experiments, we tested the hypothesis that factors associated with season, such as hormone levels or arthropod pest pressure, or with location, such as diet, temperature/humidity, or pest/predator pressure, are reflected in uropygial secretion composition. Finally we compared the composition of secretions produced by males and females to determine if uropygial secretions differ between the two sexes.

Methods and Materials

Chemical Standards The following chemicals were used as GC-MS standards: acetic acid (glacial) (Acros Organics, Geel, Belgium); benzaldehyde (99%), hexanoic acid (99.5 +%), dodecanoic acid (98%), nonadecanol (99%), tetracosanoic acid (99%) (Aldrich Chemical, Milwaukee, WI, USA); butanoic acid (99 +%), 3-methylbutanoic acid (reagent grade), octanoic acid (98 +%), nonanoic acid (97 +%), 2-tridecanone (99%), 2-pentadecanone (99%), eicosanoic acid (99 +%), docosanol (98%), docosanoic acid (99 +%), hexacosanoic acid (95 +%) (Sigma-Aldrich Chemical, St. Louis, MO, USA); pentanoic acid (technical), dodecanol (98 +%), tetradecanoic acid (98 +%), hexadecanol (99%), octadecanol (99%) (Eastman Chemical,

Kingsport, TN, USA); heptanoic acid (99 +%), decanol (99%), undecanol (98%), tridecanol (99%), tetradecanol (98 +%), pentadecanol (99%), heptadecanol (98 +%), eicosanol (97 +%) (Fluka Chemical, Milwaukee, WI, USA); hexadecanoic acid (Fisher Scientific, Pittsburgh, PA, USA); oleic acid (J.T. Baker, Phillipsburg, NJ,USA); octadecanoic acid (Matheson, Coleman, and Bell, Norwood, OH,USA).

Bird Handling We captured gray catbirds in mist nets from May through August 2009 at Killbuck Marsh Wildlife Area (40°41′N, 81°58′W), in Wayne County, Ohio, and in January 2010 at Archbold Biological Station (27°10′N, 81°21′W) in Highlands County, Florida. Upon capture, we collected 0.1 ml blood via brachial venipuncture into 300 μl of cell lysis solution to determine sex via polymerase chain reaction (PCR) based analysis of sex specific CHD genes (Griffiths et al., 1998). Birds also were weighed, measured, examined for ectoparasites, and banded. Age was determined by plumage according to Pyle (1997).

Sample Collection Uropygial secretions were collected in 1-inch lengths of glass capillary tubing (ID=1.5 mm; OD=1.8 mm; Kimble, Vineland, NJ,USA). Samples were collected by placing one end of the tubing over the gland opening and gently massaging the base of the gland until secretion emerged from the papilla and wicked spontaneously into the capillary. Collection tubes were stored in labeled and pre-weighed 1.8-ml chromatography vials that had been baked at 110°C, along with their caps and Teflon/silicon septa, for at least 8 h. Approximately 5 mg of uropygial secretion were collected from each bird sampled in summer (mean ± SD=0.005±0.001 g, N=64); approximately 3 mg of secretion were collected from birds sampled in winter (mean ± SD=0.0027±0.0008 g, N=18). Samples were stored at −80°C until analyzed.

Sample Analysis All samples first were subjected to static headspace solid-phase microextraction (SPME; volatiles analysis) and then to solvent extraction (semivolatiles analysis). In each case, extraction was followed immediately by GC-MS analysis. Between the two analyses, samples were stored at −80°C.

For analysis of volatile compounds, sealed vials containing secretion were placed into a heating block maintained at 44°C (the body temperature of gray catbirds) to equilibrate volatile chemicals in the headspace. A SPME fiber (75 μm with Carboxen-PDMS coating; Supelco, Bellefonte, PA,USA) was exposed to the headspace for 60 min, after which the fiber was retracted and the adsorbed chemicals were analyzed by GC-MS.

For analysis of semivolatile compounds, secretions were dissolved to a final concentration of 20 mg/ml in dichloromethane (DCM; trace grade; Pharmco-AAPER, Brookfield, CT,USA) containing 0.005% (v/v) benzyl alcohol (≥99%; Aldrich Chemical, Milwaukee, WI,USA) as internal standard. Samples were heated at 44°C for 30 min to dissolve, followed by centrifugation for 2 min at 14,000 × g to pellet any insoluble material.

Chromatographic Methods Separation and identification of compounds from uropygial samples used a Trace GC gas chromatograph equipped with a Polaris Q quadrupole ion trap mass analyzer (Thermo Finnigan, Waltham, MA,USA). Thermogreen LB-2 septa from Supelco were used in the injection port.

For volatiles analysis via SPME, fibers were exposed for 10 s in a 225°C injection port to desorb volatiles. Injection was in splitless mode with a 1-ml/min flow of helium. The gas chromatograph contained a polar AT-WAX column (15 m length, 0.25 mm ID, 0.50 μm film thickness; Alltech, Deerfield, IL,USA.). The column was held at 50°C for 1 min, ramped at 15°C/min to 200°C, then held at 200°C for 2 min. Detection was performed with electron ionization (EI) in full-scan, positive ion mode over a mass-to-charge ratio (m/z) range of 33–350. After each analysis, the SPME fiber was cleaned by exposing it in the heated GC injection port for at least 10 min. Prior to incubation of the fiber with any sample or standard, a blank injection was done to confirm that material from previous analyses was not carried over.

For semivolatiles analysis, 1 μl of dissolved secretion was injected manually in splitless mode with a 1-ml/min flow of helium. The gas chromatograph contained a nonpolar DB-5MS column (30 m length, 0.25 mm ID, 0.25 μm film thickness; Alltech, Deerfield, IL,USA.) Column temperature was initially 50°C followed by a ramp at 4°C/min to 90°C and a second ramp at 30°C/min to 280°C. A final ramp at 1°C/min took the column to 320°C, where it was held for 5 min. Detection of semivolatile compounds was performed in both EI and chemical ionization (CI) modes (in separate runs). EI detection was in full-scan, positive ion mode over a mass-to-charge ratio (m/z) range of 50–650. CI mode used a 1.6-ml/min flow rate of methane as the ionization gas and the same m/z range as EI mode.

Compounds were identified tentatively by searching a NIST library and by examining selected ion current chromatograms generated post-analysis in which ions characteristic of different functional groups were selected (m/z=55 for alcohols; m/z=58 for ketones; m/z=60 for carboxylic acids). Identifications were confirmed by analyzing standard compounds under identical extraction and analysis conditions and comparing retention times and fragmentation patterns.

For quantification of volatiles, use of an internal standard was not practical. Integrated peak areas from total ion chromatograms were subjected directly to the statistical analyses used for inter-group comparisons. We only

compared peak areas of the same compound across different samples, and did not compare quantities of different volatile compounds within the same sample.

For quantification of semivolatiles, integrated peak areas were normalized by dividing them by the peak area of the internal standard (benzyl alcohol) from the same chromatographic run. Normalized peak areas were subjected to the statistical analyses used for inter-group comparisons. In effect, the data were normalized twice, first by preparing secretion samples to the same concentration, and second by dividing each analyte's peak area by the internal standard peak area.

Statistical Analysis We used principal component analysis with an equamax rotation (PCA) to reduce the data to principal components (PCs) that represented correlations of peak areas of compounds found in uropygial secretions as described above. We then used multivariate analysis of variance (MANOVA) to determine the effect of sex, age, and season/location on the generated PCs and a one-way

Table 1 Compounds identified in gray catbird uropygial secretions

Volatiles identified by SPME-GC-MS			
Number	Compound	RT on AT-WAX (min)	Ion (m/z)[a]
1	Acetic acid	4.7	60
2	Benzaldehyde	5.3	77
3	Butanoic acid	6.1	60
4	3-Methylbutanoic acid	6.4	60
5	Pentanoic acid	6.9	60
6	Hexanoic acid	7.7	60
7	Heptanoic acid	8.4	60
8	Octanoic acid	9.1	60
9	Nonanoic acid	9.8	60
Semivolatiles identified by solvent extraction coupled with GC-MS			
Number	Compound	RT on DB5-MS (min)	Ion (m/z)[a]
10	Decanol	12.6	55
11	Undecanol	13.5	55
12	2-Tridecanone	14.0	58
13	Dodecanol	14.1	55
14	Dodecanoic acid	14.2	60
15	Tridecanol	14.3	55
16	Tetradecanol	14.8	55
17	2-Pentadecanone	14.9	58
18	Pentadecanol	15.0	55
19	Tetradecanoic acid	15.1	60
20	Hexadecanol	15.5	55
21	Hexadecanoic acid	15.8	60
22	Heptadecanol	15.9	55
23	Octadecanol	16.2	55
24	Oleic acid	16.3	60
25	Octadecanoic acid	16.4	60
26	Nonadecanol	16.5	55
27	Eicosanol	16.9	55
28	Eicosanoic acid	17.2	60
29	Uneicosanoic acid[b]	17.5	60
30	Docosanol	17.6	55
31	Docosanoic acid	18.1	60
32	Tetracosanoic acid	18.6	60
33	Hexacosanoic acid	19.3	60
34	C28 ester[b]	21.5	43

[a] "Ion" designates fragment ions used for preliminary identification of functional groups; [b] tentative identification

analysis of variance (ANOVA) to examine the interaction between age and sex. All statistical analyses were performed using PASW Statistics 18 (2009). A *P*-value of ≤0.05 was considered significant.

To test for the effects of sex and age on semivolatiles, only samples collected in Ohio during the summer were compared (HY males *N*=13; HY females *N*=11; AHY males *N*=22; AHY females *N*=17). To test for the effect of season/location and sex on semivolatiles, we compared AHY birds collected in Florida during the winter and in Ohio during the summer (OH males *n*=22; OH females *N*=17; FL males *N*=10; FL females *N*=7).

Similarly, to test for the effects of sex and age on volatiles, only samples collected in Ohio during the summer were compared (HY males *N*=20; HY females *N*=13; AHY males *N*=22; AHY females *N*=19). To test for the effect of season/location on volatiles, we compared AHY birds collected in Florida during the winter and in Ohio during the summer. Males and females were combined for this seasonal analysis because very few females were captured in FL during the winter (OH *N*=41; FL *N*=18).

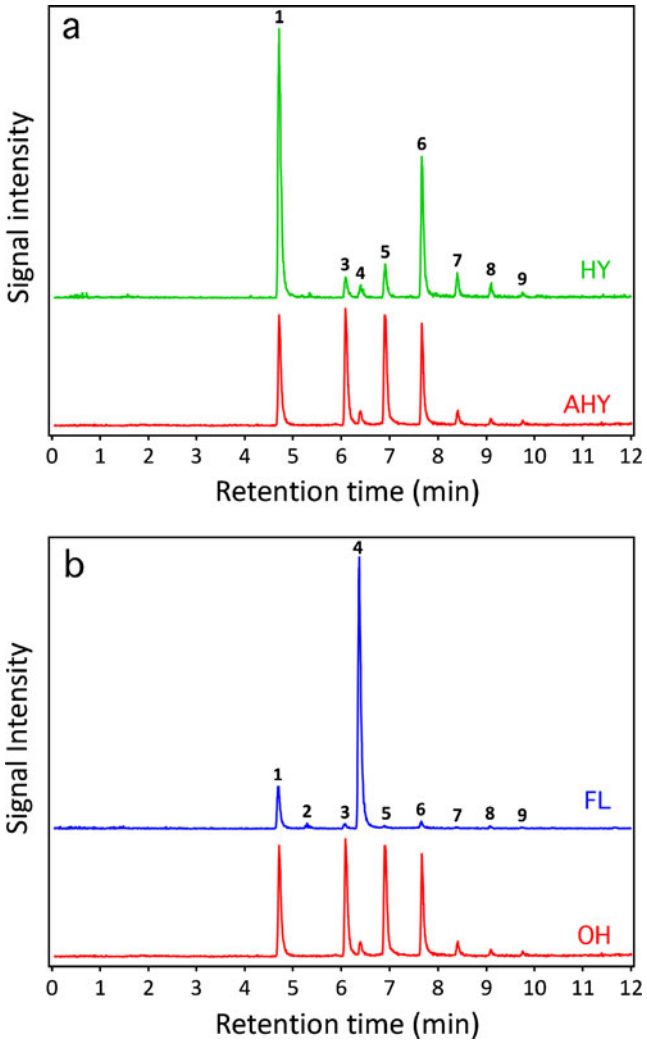


Fig. 1 **a** Gas chromatograms of uropygial secretions produced by male after hatch year (*AHY, below, red*) and hatch year (*HY, above, green*) catbirds showing the differing abundances of volatiles from birds of different age. **b** Gas chromatograms of uropygial secretions produced by male catbirds captured during the summer in Ohio (*OH, below, red*) and during winter in Florida (*FL, above, blue*) showing the differing abundances of volatiles depending on season/location. Chromatograms are shown in selected ion monitoring mode with *m/z*=60, characteristic of carboxylic acids. Peak numbers refer to Table 1

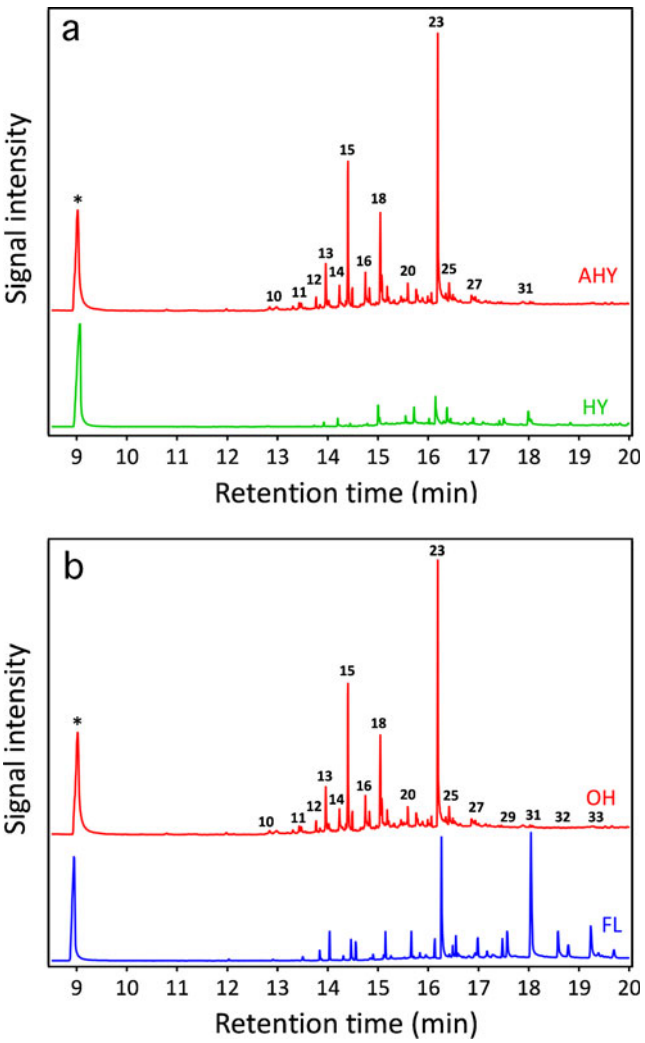


Fig. 2 **a** Gas chromatograms of uropygial secretions produced by male after hatch year (*AHY, above, red*) and hatch year (*HY, below, green*) catbirds showing the differing abundance of semivolatile compounds in birds of different age. **b** Gas chromatograms of uropygial secretions produced by male catbirds captured during the summer in Ohio (*OH, above, red*) and during winter in Florida (*FL, below, blue*) showing the differing abundance of semivolatile compounds depending on season/location. Total ion currents are shown. The peak corresponding to the internal standard (*benzyl alcohol*) is labeled with an asterisk; peak numbers refer to Table 1. Labels on minor peaks have been omitted for clarity

Table 2 Rotated component matrix for age effects on volatile compounds

Principal Component[a]			
Compound	1	2	3
Acetic acid	0.361	**0.781**	–0.229
Benzaldehyde	−0.253	0.482	0.535
Butanoic acid	**0.751**	0.510	–0.160
3-Methylbutanoic acid	0.086	**0.929**	0.245
Pentanoic acid	**0.929**	0.208	–0.005
Hexanoic acid	**0.943**	0.093	0.104
Heptanoic acid	**0.935**	0.027	0.156
Octanoic acid	0.100	0.033	**0.873**
Nonanoic acid	0.106	–0.034	**0.825**

[a] Values ≥0.7 are in bold

Table 3 Rotated component matrix for season/location effects on volatile compounds

	Principal Component[a]		
Compound	1	2	3
Acetic acid	0.403	**0.809**	–0.160
Benzaldehyde	0.051	**0.849**	0.333
Butanoic acid	**0.848**	0.354	0.045
3-Methylbutanoic acid	0.077	**0.958**	–0.009
Pentanoic acid	**0.949**	–0.016	–0.021
Hexanoic acid	**0.843**	0.177	0.319
Heptanoic acid	0.534	0.250	0.661
Octanoic acid	−0.005	0.024	**0.781**
Nonanoic acid	0.084	–0.015	**0.897**

[a] Values ≥0.7 are in bold

Results

In total, we identified 34 compounds in gray catbird uropygial secretions. Table 1 lists the identified compounds along with their retention times, type of column used for GC-MS analysis, and characteristic ion used for identification. The compounds we identified were linear and branched saturated carboxylic acids, an unsaturated carboxylic acid, linear saturated alcohols, an aromatic aldehyde, two ketones, and a 28-carbon ester. Compounds containing both odd and even numbers of carbons were identified. We also identified a series of esters at retention times greater than 22 min, but we did not attempt to characterize these esters.

Representative chromatograms from SPME/GC-MS analysis are shown in Fig. 1. We observed that the volatile profile of secretions from adult birds (designated after hatch year or AHY) were qualitatively different from the secretions of juvenile birds (designated hatch year or HY), as shown (Fig. 1a). We also observed that secretions gathered from birds in Ohio during the summer breeding season (designated Ohio or OH) differed from secretions sampled at the Florida wintering grounds (designated Florida or FL); representative chromatograms are shown (Fig. 1b). Figure 2 shows representative total ion chromatograms from DCM/GC-MS analysis. As with the volatile compounds, semivolatile compounds abundance pattern varied with age (Fig. 2a) and season/location (Fig. 2b).

We used PCA to identify sets of compounds that varied together. Table 2 shows the rotated component matrix describing the effect of age on volatile compounds; three principal components accounted for 81% of the variance in these data. The set of principal components describing season/location effects on volatile compounds is shown in Table 3. From this analysis, 82% of the variance is accounted for by three principal components. Table 4

Table 4 Rotated component matrix for age effects on semivolatile compounds

	Principal Component[a]			
Compound	1	2	3	4
Decanol	0.103	0.135	0.076	–0.163
Undecanol	−0.076	–0.154	–0.027	–0.018
2-Tridecanone	−0.017	0.454	0.188	**0.708**
Dodecanol	0.143	0.156	0.064	0.080
Dodecanoic acid	−0.017	0.310	0.641	0.562
Tridecanol	0.089	0.644	0.007	0.170
Tetradecanol	−0.029	**0.760**	0.151	0.480
2-Pentadecanone	−0.074	0.357	0.130	**0.744**
Pentadecanol	0.016	–0.291	–0.020	0.682
Tetradecanoic acid	−0.047	0.356	0.569	0.604
Hexadecanol	−0.035	0.616	0.031	0.485
Hexadecanoic acid	0.310	–0.108	**0.854**	0.044
Heptadecanol	0.080	0.378	0.181	0.191
Octadecanol	0.092	0.682	0.413	0.492
Oleic acid	0.310	0.155	0.640	0.444
Octadecanoic acid	0.628	–0.066	0.616	–0.103
Nonadecanol	0.027	**0.719**	0.163	0.084
Eicosanol	0.090	0.555	0.580	0.125
Eicosanoic acid	**0.776**	0.109	0.402	0.029
Uneicosanoic acid	**0.818**	–0.254	–0.111	0.051
Docosanol	0.200	–0.046	–0.050	–0.022
Docosanoic acid	**0.849**	0.125	0.237	–0.155
Tetraosanoic acid	**0.910**	–0.027	0.147	0.007
Hexacosanoic acid	**0.779**	0.206	0.160	0.032
C28 ester	0.158	0.344	0.621	0.228

[a] Values ≥0.7 are in bold

Table 5 Rotated component matrix for season/location effects on semivolatile compounds

Compound	Principal Component[a]			
	1	2	3	4
Decanol	0.013	–0.165	0.648	0.233
Undecanol	−0.108	–0.149	–0.255	–0.093
2-Tridecanone	−0.101	**0.877**	0.232	0.054
Dodecanol	0.016	0.045	0.388	0.144
Dodecanoic acid	−0.268	0.671	0.339	0.400
Tridecanol	−0.084	0.256	0.563	–0.036
Tetradecanol	−0.168	0.565	**0.708**	–0.012
2-Pentadecanone	−0.169	**0.909**	0.144	–0.025
Pentadecanol	0.229	0.476	–0.155	–0.012
Tetradecanoic acid	−0.257	0.621	0.338	0.481
Hexadecanol	−0.117	0.507	0.610	–0.115
Hexadecanoic acid	0.134	0.068	–0.180	**0.865**
Heptadecanol	0.009	0.199	0.296	0.105
Octadecanol	−0.124	0.655	0.620	0.283
Oleic acid	−0.112	0.570	0.205	0.542
Octadecanoic acid	0.391	–0.118	0.005	**0.770**
Nonadecanol	−0.068	0.200	0.631	0.090
Eicosanoic acid	0.432	0.015	0.210	0.623
Eicosanol	−0.109	0.128	**0.706**	0.467
Uneicosanoic acid	**0.921**	–0.120	–0.192	0.051
Docosanol	0.676	0.023	0.130	–0.024
Docosanoic acid	**0.918**	–0.144	–0.111	0.259
Tetraosanoic acid	**0.882**	–0.154	–0.166	0.164
Hexacosanoic acid	**0.901**	–0.145	–0.161	0.231
C28 ester	0.077	0.221	0.361	**0.711**

[a] Values ≥0.7 are in bold

shows the rotated component matrix for age effects on semivolatile compounds. Here, four principal components account for 59% of the variance in the data, and we note that compounds with similar functional groups tend to load onto PCs together. The compounds in PC1 are long-chain linear carboxylic acids; PC2 contains medium and long-chain alcohols; PC3 contains hexadecanoic acid; methyl ketones load onto PC4. Table 5 shows the rotated component matrix for the effects of season/location on semivolatile compounds; in this analysis the first four PCs account for 65% of the variance. Compounds with similar functional groups are again found to load onto the same PCs. Long-chain carboxylic acids load onto PC1; PC2 contains methyl ketones; PC3 contains linear alcohols; PC4 accounts for octadecanoic acid and C28 ester. Figure 3 shows score plots derived from the four PCAs. In scatter plots in which PC1 is plotted vs. PC2, the individual birds are seen to cluster based on age (Fig. 3a, c) or on season/location (Fig. 3b,d).

Age and season/location influenced levels of both volatile and semivolatile compounds (Tables 6 and 7). We found no effect of sex on signal strength of semivolatile compounds, consistent with our earlier characterization of the uropygial secretions of captive gray catbirds (Whelan et al., 2010). Sex did affect signal strength of volatile PC1. However, because we also found a significant interaction between age and sex, meaning that the effect of sex may be dependent on age, we examined the effect of sex on both AHY and HY birds separately. We found no effect of sex within AHY birds (F=0.47, P=0.499) and a significant effect of sex on HY birds (F=5.85, P=0.022, Fig. 4).

Discussion

General Observations The chemical profile we identified in gray catbird uropygial secretions resembles those reported for two other passerine species in which volatiles and semivolatiles have been characterized, i.e. dark-eyed juncos (Soini et al., 2007) and Bengalese finches (Zhang et al., 2009), in the presence of linear alcohols, carboxylic acids, and methyl ketones. Since the volatile and semivolatile chemical profiles of few passerine species have been described, it is notable that the major compounds found in these three species overlap substantially. Further characterization of the role(s) that these compounds serve for passerine species is needed.

We also note interesting differences in the chemical profile we observed. Soini et al. (2007) identified long-chain branched carboxylic acids, aliphatic aldehydes, alkanes, and alkenes as minor components in secretions of the dark-eyed junco, whereas we did not detect these compounds in gray catbird uropygial secretions. In addition, our results vary from those of Zhang et al. (2009) who identified numerous diesters with retention times between that of hexadecanol and octadecanol in the uropygial secretions of Bengalese finches. These compounds were not present in gray catbird secretions. We acknowledge that these differences may result from differences in analytical methodology. Our extraction method differs from that of Soini et al. (2007) who used sorptive stir-bar extraction, and our GC column choices differ from that of Zhang et al. (2009), who used polar columns exclusively. Inter-lab comparative analyses of samples from the same bird species would help to clarify the roles of these methodological factors. However, the observed differences also may have resulted from interspecific variation or reflect the impact of diet or other environmental factors. In both the junco and finch studies, birds were kept in captivity, whereas the gray catbirds we sampled were free-ranging. Although a more comprehensive study on the differences between captive and free-ranging birds is needed, we suggest that

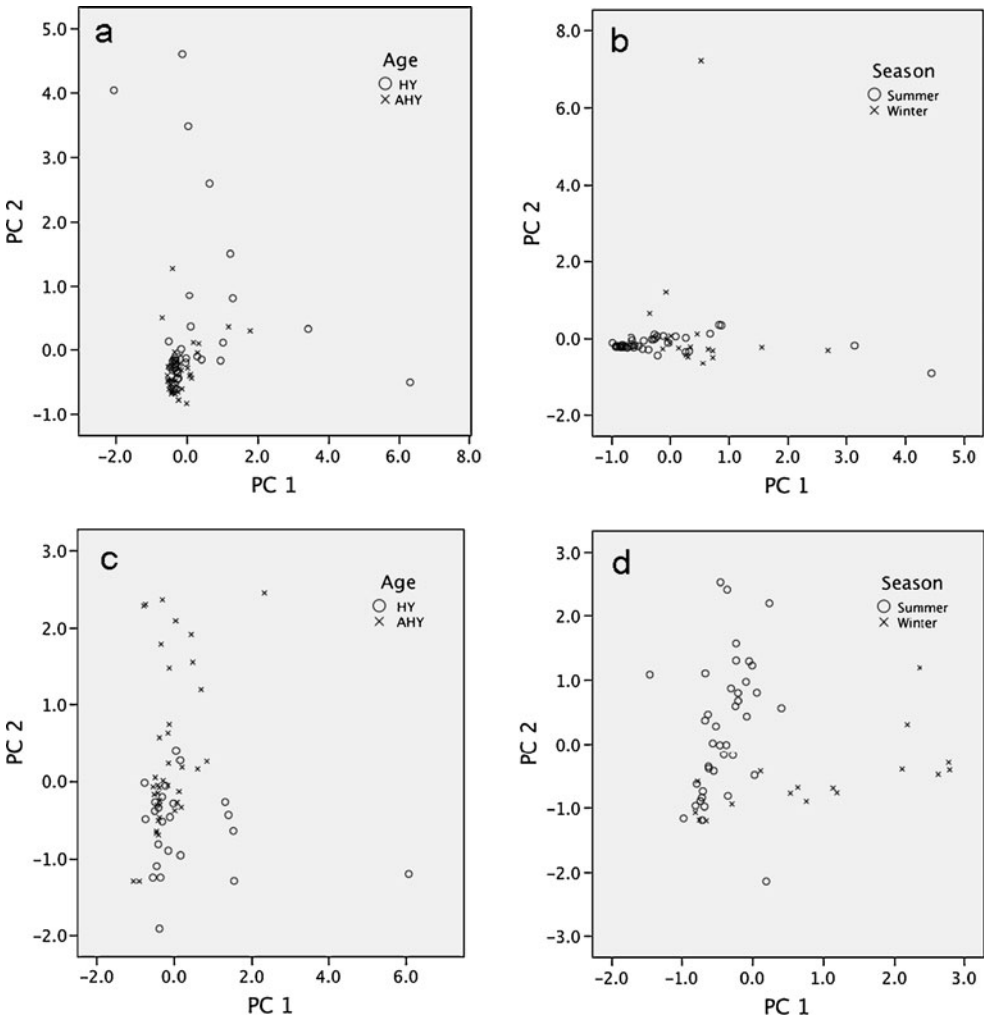


Fig. 3 Score plots resulting from principal component analysis for the effects of: **a** age on volatile compounds, **b** season/location on volatile compounds, **c** age on semivolatile compounds, and (**d**) season/location on semivolatile compounds of uropygial secretions of gray catbirds

meaningful differences in the composition of uropygial secretions may occur between captive and free-ranging birds. For example, compared with the volatile compounds we identified in captive catbirds (Whelan et al., 2010), the chemical profile of free-ranging catbirds includes greater abundance of the carboxylic acids heptanoic, octanoic, and nonanoic, fewer branched carboxylic acids, and the appearance of benzaldehyde.

Volatile Compounds The volatile compounds we identify in gray catbird uropygial secretions are saturated carboxylic acids and benzaldehyde. In the rotated component matrix for age effects on volatiles, the carboxylic acids cluster roughly based on molecular weight, with intermediate weight compounds (linear acids C4 to C7) loading onto PC1 and the heaviest compounds (octanoic and nonanoic acids) loading onto PC3. PC2 contains the lightest acid, acetic, as well as the only branched acid we detected, 3-methylbutanoic acid. The functional significance of these acids in gray catbirds has not been characterized. However, aliphatic carboxylic acids from acetic through dodecanoic have been shown to be potent oviposition repellents for

Table 6 Results of MANOVA on volatile compounds

Variable	Principal Component[a]					
	1		2		3	
	F	*P*	*F*	*P*	*F*	*P*
Age	6.113	**0.016**	10.069	**0.002**	3.746	0.057
Sex	5.353	**0.024**	0.188	0.666	0.461	0.499
Sex*age	7.627	**0.007**	0.056	0.813	0.445	0.507
Season/location	6.058	**0.017**	2.324	0.133	25.143	**0.000**

[a] Significant results ($P<0.05$) are in bold

Table 7 Results of MANOVA on semivolatile compounds

Variable	Principal Component[a]							
	1		2		3		4	
	F	P	F	P	F	P	F	P
Age	2.251	0.139	18.519	**0.000**	2.155	0.147	4.141	0.460
Sex	0.021	0.884	1.805	0.184	0.657	0.421	0.123	0.727
Sex*age	0.001	0.980	0.845	0.362	0.024	0.877	0.057	0.812
Season/location	31.709	**0.000**	7.931	**0.007**	11.108	**0.002**	0.356	0.553

[a] Significant results ($P<0.05$) are in bold

Culex and *Aedes* mosquitoes (Hwang et al., 1980, 1982; Kramer et al., 1980), consistent with an arthropod defensive function. Adult birds had low score plot values and little variation in both PC1 and in PC2, whereas hatch year birds had higher scores for volatile PC1, corresponding to higher levels of pentanoic acid, hexanoic acid, and heptanoic acid. Moreover, values of both PC1 and PC2 vary greatly among juveniles. We also observed significant interaction of sex and age with volatile PC1 for juvenile birds only (Fig. 4). Perhaps the large variation in volatile compounds among juvenile birds creates an individual olfactory signature of the sex or overall health of young birds for parents. Although our study did not explicitly include nestlings, it is reasonable to expect similar volatile chemical profiles between nestlings and juveniles, considering that some of the juveniles we sampled had only fledged a short time before. Further studies focusing specifically on the uropygial secretions of gray catbird nestlings are ongoing.

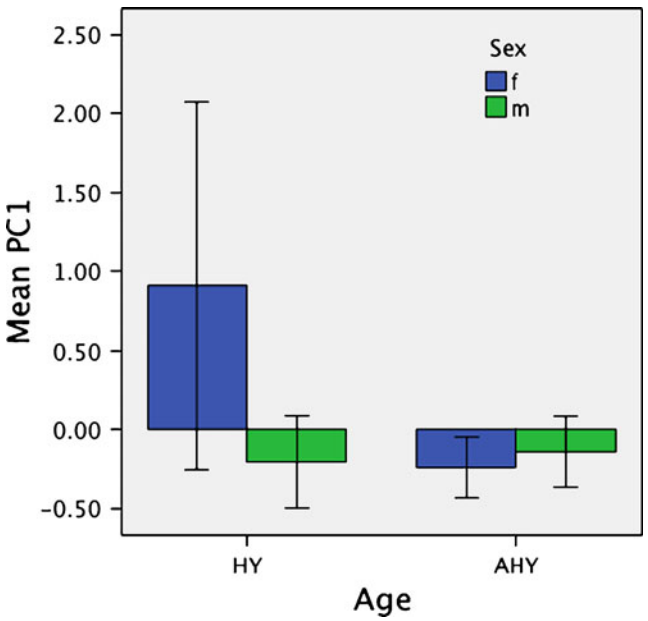


Fig. 4 Mean PC1 volatile compound values for hatch year (HY) female and male catbirds and after hatch year (AHY) female and male birds. All samples were collected in Ohio during summer. Bars indicate standard error

Although benzaldehyde does not load significantly onto any of the PCs, we find it particularly interesting in that we did not detect it in captive gray catbirds (Whelan et al., 2010), and, therefore, predict that it is sequestered from diet. Benzaldehyde is found in stone fruits such as wild black cherries (Cimprich and Moore, 1995), which are abundant at our field site during the study period. Moreover, the pits of this fruit were observed in catbird feces during this study.

In the rotated component matrix for season/location effects on volatiles, the loading onto PCs is again approximately related to molecular weight, with compounds of intermediate weight loading onto PC1, the lightest compounds loading onto PC2, the heaviest compounds loading onto PC3. An interesting exception to this trend is butanoic acid, which loads onto PC1 whereas 3-methylbutanoic acid loads onto PC2. Benzaldehyde loads onto PC2 along with acetic and 3-methylbutanoic acids. Heptanoic acid is exceptional in not loading significantly onto any of the PCs, though with a value of 0.661 it is marginally associated with PC3 and the heaviest and therefore least volatile compounds. A score plot (Fig. 3b) reveals considerable individual variation in PC1 for both winter and summer birds, whereas variation in PC2 is small for birds sampled in the summer and only marginally greater for birds sampled in the winter if the contribution of one exceptional individual is excluded. This difference may reflect variation in food resources between the two sites (see the review by Hagelin and Jones, 2007). Such dietary influences on uropygial secretions are deserving of further exploration. Alternatively, these differences also could reflect population-level genetic variations such as those reported by Whittaker et al. (2010) in dark-eyed juncos.

Semivolatile Compounds Among semivolatile compounds, we found a greater diversity of chemical functional groups, including carboxylic acids, alcohols, methyl ketones and an ester. These classes of chemical compounds have been observed in other passerines (Soini et al., 2007; Zhang et al., 2009). Compounds with similar functional groups tend to load together onto PCs, meaning that their amounts

present in secretions vary together. In the analysis based on age, PC1 contained five compounds with loading scores greater than 0.7, all of which were long-chain linear carboxylic acids; PC2 contains the linear alcohols tetradecanol and nonadecanol; the only major contributor to PC3 is hexadecanoic acid, though oleic acid and two other carboxylic acids are marginal contributors with values greater than 0.6; the ketones 2-tridecanone and 2-pentadecanone load onto PC4. Examination of a score plot (Fig. 3c) shows that values of PC1, and therefore amounts of long-chain carboxylic acids, tend to be greater in juvenile birds whereas values of PC2 (corresponding to linear alcohols) are greater in adult birds. The greater levels of long chain linear carboxylic acids in juvenile birds may assist in the "timed-release" of these less volatile compounds. Given that juvenile birds are still developing adult preening behaviors, a less volatile, more persistent uropygial secretion may be beneficial to young birds. The higher levels of heavier esters observed in hatch-year birds may be similarly important for younger birds. Soini et al. (2007) connected linear alcohol abundance with signaling of reproductive readiness and olfactory crypsis. We found that adult gray catbirds, for whom signaling to conspecifics prior to mating and protection of the nest are of great importance, display greater amounts of these linear alcohols than juveniles. Although this study was not designed to investigate individual variation or the reliability of scent profile as an individual signature, it is interesting to note that both PC1 and PC2 exhibit considerable individual variation.

In the analysis of the effect of season/location on semivolatile compounds, similar functional groups are again found to load onto PCs together. PC1 contains the heaviest linear unsaturated carboxylic acids, and these compounds are significantly more abundant in birds sampled at the Florida wintering grounds (Fig. 3d) than those sampled on Ohio breeding grounds. As suggested for the volatile compounds, perhaps this observation reflects dietary differences or population-level genetic variation. PC2 accounts for two methyl ketones, and these compounds are present at higher levels during the breeding season. Soini et al. (2007) also found that methyl ketones were present at higher levels during long days. In later work Whittaker et al. (2010) found that methyl ketones were more abundant in males and suggested that these compounds contribute to a "male-like" olfactory profile. We did not observe sex differences in semivolatile compounds among gray catbirds. PC3 contains linear alcohols while PC4 contains medium-to-long chain carboxylic acids and the C28 ester.

With the exception of oleic acid, which is monounsaturated, all the compounds we identified contain saturated alkyl chains. Unsaturated lipids rarely are found in uropygial secretions (but see Montalti et al., 2005 and Haribal et al., 2009). Jacob and Ziswiler (1982) attributed the well-documented predominance of saturated lipids to the tendency of unsaturated lipids to spontaneously oxidize under atmospheric conditions, resulting in adverse effects on feathers. Oleic acid was a minor component in the secretions we analyzed and did not load significantly onto any of the principal components, making evaluation of its role difficult. Interestingly, in a comparison of C14 to C24 unsaturated carboxylic acids, oleic acid was the most effective ovipositional repellant in *Culex quinquefasciatus* (Hwang et al., 1984). Oleic acid is sometimes called the "smell of death" because of its role as a molecule that signals death in arthropods thereby eliciting an avoidance behavior (Yao et al., 2009). We suggest that such a signal would be beneficial to catbirds and other songbirds, which are constantly subject to attack by arthropod pests to which they are exposed.

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The Fecal Odor of Sick Hedgehogs (*Erinaceus europaeus*) Mediates Olfactory Attraction of the Tick *Ixodes hexagonus*

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Abstract Parasite loads of animals vary among individuals, but the underlying mechanisms have not been fully identified. Here, we investigated whether health status of hedgehogs (*Erinaceus europaeus*) is correlated with tick burden, and whether chemical cues linked to the health status of the host mediate attraction of the tick *Ixodes hexagonus*. An ecological survey conducted over 10 years, involving 226 wild hedgehogs, revealed a strong association between health status and tick burden of hedgehogs, with healthy animals being less likely to carry ticks than unhealthy ones. Behavioral choice tests demonstrated that ticks display a preference for the fecal odor from sick hedgehogs compared with healthy ones. Chemical analysis of fecal odors using gas chromatography - mass spectrometry showed differences in the odor profile between sick and healthy hedgehogs. Sick animals tended to exhibit raised levels of the volatile aromatic heterocyclic compound indole in their feces. Ticks were attracted to indole when given the choice between indole and a solvent control. However, fecal matter from healthy hosts, with the addition of indole, was not attractive to ticks, suggesting that indole interacts with other, undetected compounds in mediating attraction. This study implies that it is the attraction to fecal odor that causes higher tick burdens in sick hedgehogs. Ticks might benefit from this preference by avoiding possible repulsion mechanisms of healthy hosts. We suggest that ticks potentially choose their host based on odor linked to the host's health status.

Key Words Infochemical · Kairomone · Parasite infestation · Animal health status · Host-parasite interaction

Introduction

Parasites and their hosts are engaged in a co-evolutionary arms race that has resulted in the refinement of specific strategies by both the parasite and its host (Jackson, 2008). Parasites need to target and infest their host species, while the host needs to repel the parasite, particularly if infestation incurs a high cost. This paper addresses the methods used by ticks that selectively target a suitable mammalian host.

Ticks are vectors of human conditions such as Lyme disease (Parola and Didier, 2001) and tick-borne encephalitis (Lindgren and Gustafson, 2001). The tick, *Ixodes hexagonus*, is associated predominantly with hedgehogs, but also is found regularly on mustelid carnivores (Page and Langton, 1996), and occasionally on dogs, cats, and humans (Estrada-Pena and Jongejan, 1999; Ogden et al., 2000). *Ixodes* species are known to cause regenerative anemia in European hedgehogs (*Erinaceus europaeus*), as well as to transmit tick-borne pathogens (Skuballa et al., 2007; Pfäffle et al., 2009).

It is usual for parasites to be aggregated within their host populations. Populations of bank voles (*Myods* (*Clethrionomys*) *glareolus*), hares (*Lepus timidus*), and roe deer (*Capreolus capreolus*) show unequal distribution of ticks (*Ixodes ricinus*) among host individuals of the same species (Tälleklint and Jaenson, 1997). In hedgehogs (*Erinaceus europaeus*), tick (*Ixodes hexagonus*) infestation levels vary among individuals (Bunnell, 2001).

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The underlying mechanisms of tick aggregation are not fully understood (Anderson and Gordon, 1982). One possible mechanism of host selection by ticks could be to target animals with reduced immune status, which are less resistant to infestation. There is some evidence to support this hypothesis. For example, cattle have been found to be differentially infested with ticks, an effect attributed to variations in the host's immunological response (Roberts, 1968). Stresses such as lactation or sickness reduce immunological resistance (Seifert, 1971). Ticks that target hosts with reduced immune status are likely to have a selective advantage over those that target hosts irrespective of host resistance. Differential infestation by ticks also could be due to different exposure risk in different habitats (Gregoire et al., 2002), variation in cues emitted by the host (Birkett et al., 2004), or to differences in behavioral (Mooring and Blumstein, 2004) or immunological repulsion by the host (Allen, 1973).

Ticks employ three methods to locate hosts, namely, sitting and waiting in the open, dwelling in host nests or burrows, and active searching (Yoder et al., 1999). *Ixodes hexagonus* is thought to be a nidicolous tick that occupies the nests of hedgehogs. However, dogs and cats show significant levels of attachment by *I. hexagonus* when allowed access to urban parks (Ogden et al., 2000), thus showing that this tick also frequents areas of vegetation away from nests. Here, we focused on the sensory cues leading to attraction of ticks to their hedgehog hosts.

Ticks are attracted to hosts from a distance by using different stimuli including vibrations, body heat, and odor (Parola and Didier, 2001; Donzé et al., 2004). Host odors (kairomones) are the most specific distance stimuli utilized by arthropod parasites (Osterkamp et al., 1999). Ticks possess a cluster of 10 olfactory and gustatory receptors, known as Haller's organ, that are sensitive to vertebrate-associated chemicals including fatty acids and carbon dioxide (Osterkamp et al., 1999). Carbon dioxide, released in the breath, stimulates questing behavior (Waladde and Rice, 1982; Sonenshine et al., 1986; Osterkamp et al., 1999). It also stimulates some species to move towards the source (McMahon and Guerin, 2002). However, carbon dioxide traps have proved largely unsuccessful in attracting *Ixodes* spp. in the field (Schulze et al., 1997), suggesting that other attractants are involved (Crooks and Randolph, 2006). Kairomones that attract ticks may include volatile compounds released by glands (Carroll, 1998) or metabolic residues originating in the gut (Donzé et al., 2004) of the potential host.

Odor cues also can reveal information to a receiver about the health status of the sender (Wyatt, 2003). For example, female screwworm flies (*Cochliomyia hominivorax*) orientate to volatiles produced by a bacterium present in wounds associated with myiasis (flystrike) to find their cattle host (Hammack et al., 1987). Metabolic by-products inadvertently produced by a disease or immune response can result in odor cues that provide a reliable indicator of health and disease (Penn and Potts, 1998). Female mice (*Mus musculus*) discriminate between the odors of parasitized and non-parasitized males (Kavaliers and Colwell, 1992). It has been suggested that the odor of male mice signals infection due to a change in the composition of commensal microbes caused by the infection itself (Penn and Potts, 1998).

We hypothesized that differential tick infestation levels in hedgehogs are linked to the health status of the host, and that this is mediated by odor cues that attract or repel ticks. We tested this hypothesis by employing a multidisciplinary approach that included an ecological survey, behavioral assays, and chemical analysis. In the survey we monitored tick burden of wild hedgehogs in relation to health status. The behavioral bioassay investigated whether fecal odors mediate differential attractiveness of sick and healthy hosts. Analytical techniques complemented by behavioral assays identified a key chemical involved.

Methods and Materials

Ecological Aspects Over a period of 10 years, 226 wild hedgehogs (*Erinaceus europaeus*) were treated at a hedgehog rescue center run by T. Bunnell. Hedgehogs were brought to the center by members of the public from a 25-mile radius around York, North Yorkshire, England, UK. The main reason for them being identified as in need of care was the presence of the normally nocturnal hedgehog in the open during daylight hours. Such animals were either sick or injured and required treatment. Apart from daylight activity the animals were not subject to any selection criteria. Sampled hedgehogs included sick and healthy animals. Hedgehogs were considered sick (N=119) when they suffered from one or more ailments (Table 1) apart from tick infestation. Animals with no apparent ailments, apart from tick infestation, were considered to be healthy (N=107).

Chemical Analysis Fecal samples were collected from 31 sick and 31 healthy hedgehogs ranging in weight from 250 g to 900 g. The ailments suffered by the sick animals were noted (Supplementary Material Table 1). Fecal samples weighed between 1.03 g and 16.34 g and were stored in glass containers at −20°C. Following thawing, 0.5 g feces were placed in a sealed 5 ml glass vial for 20 min to extract head space volatiles. Solid phase microextraction was conducted using a Supelco™ SPME Portable Field Sampler with a 100 μm PDMS (polydimethylsiloxane) fiber. The chemically adsorbent fiber was

Table 1 Ailments observed in sick hedgehogs in relation to tick infestation

Ailment	Diagnosis	Total number of hedgehogs with this ailment	Percentage of hedgehogs with this ailment that were infested	Number of tick-infested hedgehogs with this ailment	Percentage of tick-infested hedgehogs with this ailment	Percentage of uninfested hedgehogs with this ailment	*P*
Crenosoma striatum	microscopic examination of feces	15	53%	8	17%	4%	<0.001
Coccidiosis (Isospora sp.)	microscopic examination of feces	7	29%	2	4%	3%	0.4
Tapeworm	microscopic examination of feces	14	50%	7	15%	4%	<0.001
Diarrhea	liquid feces	29	28%	8	17%	12%	<0.01
Sarcoptic mange	microscopic identification of skin mites	8	0%	0	0%	4%	1.00
Demodectic mange	microscopic identification of skin mites	4	25%	1	2%	2%	0.7
Ringworm	skin lesions	8	25%	2	4%	3%	0.3
Injured	signs of trauma	26	35%	9	19%	10%	<0.001
Breathing difficulties	chest sounds and/or rapid breathing	17	47%	8	17%	5%	<0.001
Malnourished	tapered rear end	54	33%	18	38%	20%	<0.001
Dehydrated	loose skin on abdomen	22	36%	8	17%	8%	<0.001
Hypothermia	abdomen cold to the touch	22	23%	5	10%	10%	<0.07
Hyperactivity	continuous movement during daylight	12	50%	6	13%	3%	<0.001
Collapsed	uncurled and immobile	5	60%	3	6%	1%	<0.02

Diagnoses were confirmed by the Minster Veterinary Practice (York, UK). Microscopic examination of fecal smears were used to identify excessive burdens of internal parasites. Percentages were derived from total numbers of infested hedgehogs (N=48) and uninfested hedgehogs (N=178), respectively. Some animals had more than one ailment. Tick infestation of hedgehogs with an ailment was compared to infestation of healthy hedgehogs using Fisher's exact test (p values were corrected for repeated testing using Hochberg's, 1988, method)

exposed for 15 min to the head space. Exposure time of 15 min was sufficient to reach equilibrium, and is comparable to that used in similar studies (Garner et al., 2007). SPME fibers were analyzed by split-less direct injection using a GC-MS (Agilent 6890 GC/5972) with a 0.25 μm x 30 m HP-5MS capillary column. Helium was the carrier gas with a flow rate of 0.8 L/min. Initial column temperature was 50°C increasing at a rate of 10°C/min to 290°C, which was held for 10 min (inlet temperature 250°C). The MS transfer line was set at 280°C, the electron multiplier voltage at 1788 V, and the energy of ionizing electron was 70 eV. Volatiles were identified tentatively by using the MS Library (NISTO2) supplied with the GC-MS software (Supplementary Material Table 2). The structures were validated with synthetic standards obtained from Sigma-Aldrich Company Ltd. and comparing retention times and MS spectra.

General Procedure for Behavioral Assays On arrival at the rescue center, the weight and health status of the hedgehogs were recorded (Table 1), and all ticks were removed. Ticks were removed by using narrow, curved-ended forceps to grasp the head end and then by turning the forceps swiftly. This caused the tick mouth parts to be removed without incurring damage to the tick or to the spines of the hedgehog. No change in behavior was observed, and all ticks in all bioassays underwent the same procedure. We used ticks removed from hedgehogs rather than collecting them in the field due to the limited availability of free-living ticks in the field.

Individual ticks were selected for testing based on their morphological integrity, such as presence of all appendages and mobility, and the display of questing behavior. Questing refers to a typical posture adopted by ticks whereby the first pair of legs is extended and they wave in response to stimuli that indicate the presence of a host (Osterkamp et al., 1999). Only hungry, questing, adult female ticks were used. Ticks were all identified as *I. hexagonus* by virtue of the positioning of the genital aperture opposite the third coxae (Hillyard, 1996). Inevita-

bly, ticks used in these experiments came from sick hedgehogs as, with the exception of a few individuals, all ticks were removed from sick animals (Fig. 1). Ticks were never tested with the feces of their individual hosts.

Following removal from hosts, the semi-gorged adult female ticks were maintained at ambient temperatures of 20–23°C and humid conditions, and were deprived of a blood meal for at least 1 d prior to being used in a bioassay. The average weight of ticks used was 0.036 g. Choice tests were conducted with a glass T-maze (25 cm trunk and 25 cm arms, internal diam, 0.9 cm) placed horizontally. The two arms of the T-maze, which constituted the top of the 'T', were exposed equally to light to avoid any bias due to negative phototaxis. Odor stimuli were randomly positioned at the end of either the left or right arm of the T-maze. Ticks were placed near the top of the trunk that formed the base of the T-maze, and the tube behind them was blocked. This prevented ticks from returning to the trunk of the maze, thus providing two possible directions of movement (left or right). Each tick was introduced separately into the T-maze, thus avoiding contact with other ticks, and no tick was ever used more than once in any trial in any of the bioassays.

Between trials, the T-maze was cleaned thoroughly with ethanol. A tick was considered to have chosen a particular arm once it had crossed the mid-point on one of the two arms. If a tick had not reached the end of either arm after 10 min, the experiment was discontinued. Ticks began to quest as soon as they were released into the top of the trunk of the maze. All bioassays were conducted within a 2 week period. Ticks were filmed during the bioassays, and their choices were scored by an observer blind with respect to stimulus location.

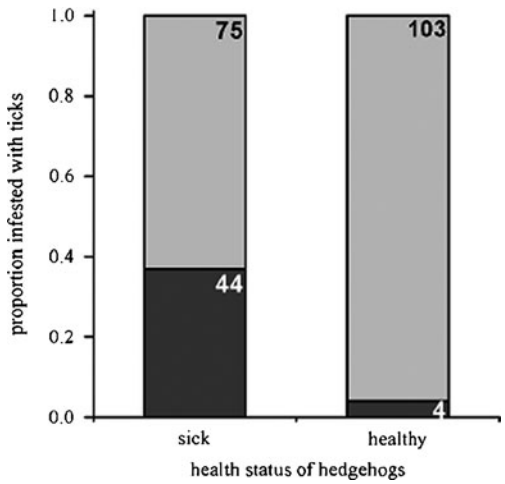


Fig. 1 Proportion of sick and healthy animals infested with ticks. Black bars denote tick-infested animals. Grey bars denote uninfested animals. The numbers of hedgehog individuals are indicated in the corner of each bar. Sick hedgehogs (N=119) were more likely to carry ticks than healthy ones (N=107; Pearson *Chi-square* test, χ^2=37.2, P<0.001)

Bioassay 1 Fecal samples were collected in the morning, having been produced by a hedgehog the previous night. They were mixed immediately with a glass rod to produce a homogenous mix, and were frozen at −20°C and kept at this temperature until needed for chemical analysis or bioassays. A different tick was used in each trial with two different fecal samples, one taken from a sick hedgehog, the other from a healthy one. No fecal sample was used more than once. In no instance were ticks collected from an individual hedgehog used in a bioassay with feces collected from the same animal. Also, in order to avoid a possible family odor signature, fecal samples from related hedgehogs were not used in the same trials.

A freshly thawed fecal pellet (0.1 ml) was placed in a Gilson tip at the end of each of the two arms forming the 'T' of the T maze. One fecal pellet was from a healthy individual, and the other from a sick one. The positioning of the pellets at the different ends of the horizontal arms of the maze was randomly determined by throwing a die. A tick was released to the top of the trunk 10 sec after the pellets were placed in position.

Bioassay 2 The procedure was identical to that used in Bioassay 1 with the exception that the ticks used were obtained from a domestic ferret (*Mustela putorius furo*). Ticks had not been exposed to the odor of sick hedgehogs and could be considered naïve for the purpose of the bioassay.

Bioassay 3 Individual ticks were placed separately at the top of the trunk of the maze. The end of one arm contained 0.04 ml indole (Sigma-Aldrich Company Ltd.) dissolved in a solution of water and ethanol (40 mg in a solution of 98% water and 2% ethanol), and the other arm contained 0.04 ml of the solvent (2% ethanol, 98% water). The concentration of indole used was determined by analyzing fecal samples with the GC-MS (Snellings et al., 2003) and was based on the amount found present in feces from sick hedgehogs.

Bioassay 4 Fecal samples collected from healthy hedgehogs were used. As with all other bioassays, a different tick was used in each trial, and no fecal sample from a particular hedgehog was used more than once for individual trials. In no instance were ticks collected from an individual hedgehog used in a bioassay with feces collected from the same animal. A freshly thawed fecal pellet (0.1 ml) from the same healthy hedgehog was placed in a Gilson tip at the end of each of the two arms forming the 'T' of the maze. The fecal pellet at the end of one arm was spiked with 0.04 ml of indole (Sigma-Aldrich Company Ltd.) dissolved

in a solution of water and ethanol (40 mg in a solution of 98% water and 2% ethanol), and the fecal pellet in the other arm was spiked with 0.04 ml of the solvent (2% ethanol, 98% water). The positioning of the pellets (whether spiked with indole or the solvent) at the different ends of the horizontal arms of the maze was randomly determined by throwing a die. A tick was released to the top of the trunk 10 sec after the pellets were placed in position.

Results

Hedgehogs typically arrived at the rescue center between May and January (Bunnell, 2009). On arrival, 21% (N=48) of all hedgehogs (N=226) were infested with *Ixodes hexagonus* ticks. Of the infested individuals, 44 were sick and 4 were healthy (Fig. 1). Overall, sick hedgehogs were more likely than healthy ones to carry ticks (Pearson *Chi-Square* test, χ^2=37.2, P<0.001; Fig. 1). Hedgehogs suffering from any of 12 different ailments including lungworm (*Crenosoma striatum*), tapeworm, ringworm, breathing difficulties, hypothermia, and injury were more likely to carry ticks than healthy hedgehogs (Pearson *Chi-Square* tests, see Table 1). Only 4 of the hedgehogs carrying ticks displayed no obvious ailments, 3 of these being orphaned nestlings (Fig. 1). Some animals suffered from combinations of ailments (Supplementary Material Table 3).

The association between tick infestation and presence of other ailments could result either from ticks targeting hedgehogs that are already sick or from ticks targeting healthy animals that then become sick due to the tick infestation. However, some of the ailments suffered by sick hedgehogs (Table 1) were not caused by ticks. These include injuries from other animals, machinery, or road traffic, bacterial infections, heavy intestinal parasite burdens resulting from ingestion of certain food sources, and hypothermia caused by prolonged exposure to low temperatures. We tested the hypothesis that ticks are attracted to the feces of hedgehogs that are already sick.

In bioassay 1, more ticks (obtained from hedgehogs) chose the arm in a T-maze containing feces from a sick hedgehog (N=16) than the arm containing feces from a healthy hedgehog (N=4; binomial test, binomial proportion 0.2, P<0.012). In bioassay 2, more ticks (obtained from a ferret) chose the arm in a T-maze containing feces from a sick hedgehog (N=13) than the arm containing feces from a healthy hedgehog (N=4; binomial test, binomial proportion 0.24, P<0.049).

Individual fecal odor profiles of 62 hedgehogs (31 sick, 31 healthy) were analyzed. Out of 7 volatile compounds identified in the odor profiles (Supplementary Material Table 2) only one substance, indole, was present in significantly higher amounts in sick hedgehogs compared with healthy ones (Wilcoxon test, χ^2=19.6, P<0.001; Fig. 2). The presence of indole was confirmed by using a standard and included exact matches of chromatographic and mass spectral data, taken under equivalent concentration under the same operating conditions.

In bioassay 3, ticks were attracted to indole when given a choice between indole and the solvent control (2% ethanol, 98% water). They chose the arm of the T-maze containing indole significantly more times (N=17) than the arm with the control (N=3; binomial test, binomial proportion 0.15, P=0.003). In bioassay 4, ticks displayed no significant preference when given a choice between two samples of healthy feces from the same hedgehog, spiked respectively with indole (approached by 7 ticks) and the solvent (approached by 7 ticks; binomial test, binomial proportion 0.5, P=1.0).

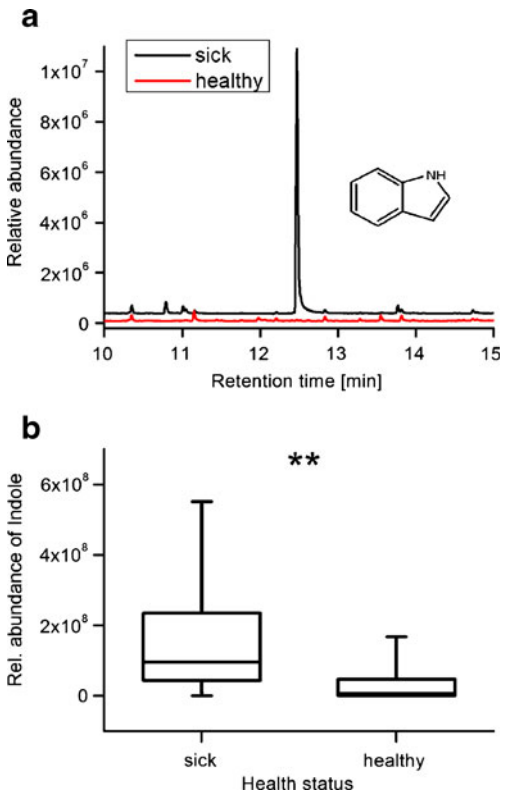


Fig. 2 Abundance of indole in hedgehog fecal odor. Abundance was measured by head space sampling using Solid Phase Microextraction. **a** Examples of gas chromatograms from a typical sick (top line) and a typical healthy (bottom line) individual (Agilent 6890 GC/5972 MS with a 0.25 μm×30 m HP-5MS capillary column; identification by MS Library NISTO2). For clarity, we offset the chromatogram of the sick hedgehog by 300,000 units. The peak represents level of indole in the sick hedgehog. **b** Relative abundance of indole in sick (N=31) and healthy (N=31) hedgehogs. The range indicated by the boxes is from 25 to 75 percentiles and includes the median. Error bars denote 5 and 95 percentiles (error bars) of relative abundance of indole in chromatograms. Asterisks denote significant differences (Wilcoxon test, χ^2=19.6, P<0.001) between the two health states

Discussion

This study sheds light on the mechanisms of parasite host interaction between ticks and hedgehogs. Sick hedgehogs were infested by a significantly higher number of ticks than healthy ones. This initial finding might be due to the preference of ticks for sick hosts. Alternatively, tick infestation could be the cause of illness. Potentially, ticks attached to a hedgehog also might attract more ticks through tick-tick attraction. While we cannot exclude a synergistic effect of these alternative mechanisms, the attraction of ticks to the feces of sick hedgehogs, rather than to the feces of healthy ones suggests that ticks selectively target sick hosts.

By using ticks that had previously infested sick hedgehogs (bioassay 1) we could potentially have biased our sample of experimental animals. These ticks could perhaps show a bias towards sick hosts. However, a second bioassay, using naïve ticks obtained from a domestic ferret, showed that these ticks also displayed a preference for the feces of sick hedgehogs rather than the feces of healthy animals.

The aromatic organic compound indole in the feces (Fig. 2) was the only major odor compound occurring in larger amounts in fecal odor from sick hedgehogs compared to fecal odor from healthy ones. Bioassay 3 showed that ticks are attracted to synthetic indole alone and hence that indole is likely involved in the attraction of ticks to sick hedgehogs.

The lack of preference of ticks for indole when it was used to spike feces from healthy hedgehogs (bioassay 4) might be explained by the masking or repellent compounds in the fecal odor of healthy hedgehogs. A range of other odor compounds was detected in the fecal odor of healthy hedgehogs compared with that of sick ones. These substances potentially could serve to repel ticks, while also masking the effect of any attractive substance such as indole. We cannot exclude the possibility that other minor compounds in the odor of feces of sick hedgehogs also may serve to attract ticks. However, indole was the only substance found present in significantly higher amounts in the feces of the sick hedgehogs compared to healthy ones. Future studies should test such potential synergistic effects.

Why should ticks target sick hedgehogs in preference to healthy ones? Tick infestation triggers a cell-mediated immune response in some host species leading to rejection or to death of the tick (Brown and Askenase, 1985). Acquired resistance of guinea pigs to tick (*Amblyomma americanum*) feeding is mediated by cellular walling off by employing basophils, which obstruct access to blood in the lower dermis (Allen, 1973). Cellular walling off results in reduced tick numbers and weight, or even death of the tick *in situ.* This finding in tick-infested guinea pigs provides some indication of how the mechanism might work in other animals. Mice that are stressed or sick have a depressed immune system making them more susceptible to infection (Kiank et al., 2006). They become more lethargic and less able to protect themselves from mosquitoes (Edman and Day, 1983).

Why would ticks use fecal odor to locate their hedgehog host? Apart from anal glands and sweat glands under the feet, hedgehogs have no specialized skin glands (Reeve, 1994). Odor sources that attract hedgehog antagonists from a distance, therefore, may be limited to footprints and feces. As the tick *I. hexagonus* appears to dwell in open vegetation as well as in nests, fecal deposits could provide a cue that enables ticks to target their hedgehog hosts in areas where hedgehogs are foraging and are either stationary or slow moving. Hedgehogs also tend to use similar routes while away from the nest, and are likely to pass by feces deposited previously. Hence, hedgehog fecal deposits are a good place for ticks to encounter a passing host.

Once near the odor source, some *Ixodes* species ambush their host by waiting on or near the chemical residues left by the host (Rechav et al., 1978; Carroll et al., 1995). Ticks such as *I. scapularis* aggregate in places associated with host presence (Carroll et al., 1995; Carroll and Schmidtmann, 1996; Carroll, 2002), thus increasing their chances of encountering a host that is likely to visit these areas on a regular basis. Near field stimuli such as body heat, vibrations or carbon dioxide that originate from the host facilitate final contact of the tick with the host (Parola and Didier, 2001).

Fecal products can provide information regarding physiological condition of a host, including the general health and the presence of enteroparasites (Patton et al., 1986). Residues from the digestive tract of hosts attract ticks. For example, ungulates release volatile metabolites from the rumen that are carried on the air and attract *Ixodes* spp. from a distance (Donzé et al., 2004).

Indole, identified here in the fecal odor of sick hedgehogs (Fig. 2) is present in odor profiles of other mammals, e.g., the skin and feces of giraffes (Wood and Weldon, 2002) and in the volatile headspace of incubated human sweat (Meijerink et al., 2000). The mosquito (*Anopheles gambiae*) possesses an odorant receptor that is finely tuned to a set of aromatic compounds that includes indole (Carey et al., 2010). Indole is produced in the human gut by bacterial decomposition of the amino acid tryptophan (Jensen et al., 1995). In humans, an abnormal balance of intestinal bacteria is related directly to diseases and immunological disorders, and is indicated by increased levels of indole (Fukuuchi et al., 2002). Future studies will aim to identify the chemical nature of repellent compounds possibly present in odor of feces released by healthy hedgehogs. The combined use of attractants and

repellents could be applied to the environmental management of ticks, as has been shown successfully in insects (Chapman et al., 1998).

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Anointing Chemicals and Hematophagous Arthropods: Responses by Ticks and Mosquitoes to Citrus (Rutaceae) Peel Exudates and Monoterpene Components

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Abstract Some birds and mammals roll on or wipe themselves with the fruits or leaves of *Citrus* spp. or other Rutaceae. These anointing behaviors, as with anointing in general, are thought to function in the topical acquisition of chemicals that deter consumers, including hematophagous arthropods. We measured avoidance and other responses by nymphal lone star ticks (*Amblyomma americanum*) and adult female yellow fever mosquitoes (*Aedes aegypti*) to lemon peel exudate and to 24 volatile monoterpenes (racemates and isomers), including hydrocarbons, alcohols, aldehydes, acetates, ketones, and oxides, present in citrus fruits and leaves in order to examine their potential as arthropod deterrents. Ticks allowed to crawl up vertically suspended paper strips onto a chemically treated zone avoided the peel exudate and geraniol, citronellol, citral, carveol, geranyl acetate, α-terpineol, citronellyl acetate, and carvone. Ticks confined in chemically treated paper packets subsequently were impaired in climbing and other behaviors following exposure to the peel exudate and, of the compounds tested, most impaired to carveol. Mosquitoes confined in chambers with chemically treated feeding membranes landed and fed less, and flew more, when exposed to the peel exudate than to controls, and when exposed to aldehydes, oxides, or alcohols versus most hydrocarbons or controls. However, attraction by mosquitoes in an olfactometer was not inhibited by either lemon peel exudate or most of the compounds we tested. Our results support the notion that anointing by vertebrates with citrus-derived chemicals deters ticks. We suggest that some topically applied compounds are converted into more potent arthropod deterrents when oxidized on the integument of anointed animals.

Key Words Citrus · Ectoparasites · Ticks · Mosquitoes · Monoterpenes · Anointing · Repellent

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Introduction

Many birds and mammals roll on or wipe themselves with fruits, leaves, or other plant parts (reviewed in Weldon and

Carroll, 2007). Some animals incorporate aromatic leaves or bark in their nests. Phytochemicals appropriated via these anointing and fumigation behaviors are believed to deter consumers, including nuisance arthropods.

Passerine birds, including grackles (*Quiscalus* spp.) (Laskey, 1948; Johnson, 1971; Clayton and Vernon, 1993), starlings (*Sturnus vulgaris*) (Hampe in Stresemann, 1935), and tanagers (e.g., *Tachyphonas coronatus* and *Tangara mexicana*) (Sick, 1993), and mammals, including capuchin (*Cebus* spp.) (Buckley, 1983; Baker, 1996; Leca et al., 2007) and spider monkeys (*Ateles geoffroyi*) (Richard, 1970), wolves (*Canis lupus*) (Zimen, 1981), and coatis (*Nasua* spp.) (C. Cranmore, pers. comm.), anoint with lemons (*Citrus limona*), limes (*Citrus aurantifolia*), or other citrus fruits (Rutaceae), applying the peels, pulp, and/or juice to their integument (Fig. 1). White-faced capuchin monkeys (*Cebus capucinus*) in Costa Rica fur-rub more frequently with citrus fruits, which are introduced in the Americas, than with native plants (Baker, 1996). Spider monkeys in Panama also fur-rub with the leaves of rutaceous trees (*Citrus* and *Zanthoxylum*) (Campbell, 2000).

Humans have long used the Rutaceae as a source of arthropod deterrents. Ancient Europeans placed the fruits and leaves of lemon and other citrus plants among stored garments to deter moths (Arias and Ramón-Laca, 2005). In both the New and Old Worlds, the ethnobotanical uses of the Rutaceae include the topical application of fruit and leaf extracts to repel biting insects (Anaso et al., 1990; Koh and Ong, 1999; Arias and Ramón-Laca, 2005). Sukumar et al. (1991) regard the Rutaceae to be among the most promising of plant families to yield mosquito control agents.

Many volatiles present in citrus tissues possess biocidal and other arthropod-deterrent properties, an observation consistent with the defensive activity hypothesized for these substances in the context of vertebrate anointing. A study of the fumigant activity of 30 citrus peel volatiles against adults of the cowpea weevil (*Callosobruchus maculatus*), a stored product pest, for example, indicated that the chief component, limonene, and 21 other compounds are insecticidal (Don-Pedro, 1996). An investigation of the contact miticidal activity of 30 monoterpenoids against the two-spotted spider mite (*Tetranychus urticae*), a plant-infesting pest, found citronellol, carvone, and geraniol among the most effective compounds (Lee et al., 1997). These or other volatile monoterpenes also repel or kill arthropods that molest tetrapods, including ticks (Chungsamarnyart and Jansawan, 1996; Thorsell et al., 2006; Del Fabbro and Nazzi, 2008; Pälsson et al., 2008), mites (Carroll, 1994; Perrucci et al., 1995), lice (Toloza et al., 2006), fleas (Hink et al., 1988), bugs (Safra et al., 2009), and midges (Logan et al., 2009) and other biting flies (e.g., Zaki et al., 2005; Butler, 2007).

Clayton and Vernon (1993) investigated the potential value of grackles' habit of anointing with limes by conducting experiments with the chewing louse, *Columbicola columbae*, a feather-feeding ectoparasite that impairs host survival and mating success. Lice confined in Petri dishes with lime slices experienced higher mortality than did those confined with water. Lime pulp juice brushed onto lice had little effect, but the peel extract was insecticidal.

We tested nymphs of the lone star tick (*Amblyomma americanum*), a vector of human monocytic ehrlichiosis in the United States, and adult females of the yellow fever mosquito (*Aedes aegypti*), a circumtropical vector of yellow



Fig. 1 Convergence in anointing with citrus fruit by New World vertebrates. (*left*) A tufted capuchin monkey (*Cebus apella*) rubs a lemon slice against its back. (*center*) A boat-tailed grackle (*Quiscalus major*) rubs a lemon slice against its wing and trunk. (*right*) A white-nosed coati (*Nasua narica*) rubs a lemon slice against its tail

fever and dengue viruses, filariasis, and other diseases, for responses to lemon peel exudate and to the chief volatile monoterpenes reported in citrus fruits and leaves. Bioassays were conducted in order to evaluate these substances as ectoparasite deterrents for animals that anoint with them. Both arthropods were tested with 24 monoterpenes (racemates and isomers), including hydrocarbons, alcohols, aldehydes, acetates, ketones, and oxides.

Tick host-finding behavior ranges from ambushing to hunting, depending upon the species (Waladde and Rice, 1982). When stimulated by chemical or other cues, lone star ticks leave ambush sites and crawl toward stationary or slow-moving hosts. After locating a suitable site on the host, they typically feed for days. Mosquitoes, in contrast, swiftly fly toward and land on hosts, imbibing a blood meal within seconds before departing. We tested these distantly related and behaviorally disparate hematophagous arthropods in assays appropriate to their respective methods of host acquisition.

Arthropods in each assay were tested with fresh (unconcentrated) lemon peel exudate and with individual monoterpenes. Ticks in a repellent experiment were tested for their tendency to avoid a substrate treated with peel exudate or monoterpenes in order to assess how they respond when encountering a scent-laden host. Ticks in a toxicity experiment were tested for their motor performances and host-acquisition responses following confinement with these substances, as might occur when they infest a host's integument where the plumage or pelage traps volatiles. Mosquitoes in a feeding experiment were confined in a module with chemically treated membranes to evaluate their landing, feeding, and flying responses to peel exudate or monoterpenes. Finally, mosquitoes in an olfactometer experiment were presented with an airsteam containing these substances in order to assess their anemotactic responses.

Methods and Materials

Chemicals Arthropods in each assay were tested with a fresh exudate of an organically grown lemon, obtained by lacerating a 6×6 cm area of the peel with a razor blade and pressing the exuded fluid against the test medium. Organically grown kale (*Brassica oleracea*) (also lacerated) and blank (untreated) conditions served as controls.

Ticks and mosquitoes were tested for responses to the following compounds, which typically occur in citrus peels and/or leaves (Dugo et al., 2002; Lota et al., 2002): *p*-cymene, bisabolene, (+)-limonene, myrcene, α-pinene, ß-pinene, γ-terpinene, terpinolene, valencene, citral, citronellal, (−)-carveol, citronellol, geraniol, linalool, 4-terpinenol, α-terpineol, (+)-carvone, (−)-carvone, (+)- limonene oxide, (−)-limonene oxide, citronellyl acetate, geranyl acetate, and linalyl acetate. Compounds were dissolved in 0.05 M acetone solutions for feeding membrane tests with mosquitoes and 0.1 M solutions for assays with ticks; acetone alone served as a control. A previous study indicated that the chemical concentrations used in our tests were appropriate to distinguish the effects of different compounds (Kramer et al., 2009); the concentrations of compounds deposited on anointing animals are unknown, but they will vary with the materials used, the duration of anointing, and other factors.

Arthropods Ticks from a colony at the USDA, ARS, Knipling-Bushland U. S. Livestock Insects Laboratory, Kerrville, Texas, were maintained as described by Carroll et al. (2005) and Kramer et al. (2009). At the time of testing, ticks had been in the nymphal stage for 2–6 months.

Mosquitoes for membrane-feeding tests were maintained as described by Weldon et al. (2006). Adult nulliparous female mosquitoes (Liverpool strain), 5–16-d-old, were fed a 10% sucrose solution presented on cotton pads. The sucrose-soaked pads were replaced with water-moistened pads 48 h before testing. Mosquitoes (Orlando strain) for the olfactometer test were maintained as described by Kline et al. (2003). Bioassays were conducted up to four times per day (0700, 0900, 1100, and 1300 h). Female mosquitoes, 6–12-d-old, were a fed a sugar solution on cotton pads that were removed 1 h before testing.

Bioassays with Ticks

Avoidance Test We investigated repellency by exploiting the tendency of ticks to climb, a key host-seeking behavior, inducing them to crawl onto a chemically treated zone on paper strips (Kramer et al., 2009). Ticks were tested on 1× 8 cm strips of recycled bond paper marked at 1–6 cm intervals. In an experiment of the responses of ticks to lemon peel exudate, the paper strips were covered by a piece of tape up to the 2 cm mark. The lacerated area of a lemon peel was applied by manually pressing it against the strip above the 2 cm mark. Kale was applied similarly to paper strips; untreated strips served as an additional control.

In an experiment on the repellency of individual compounds, 12 µl of each solution or plain acetone were evenly applied by a micropipette to paper strips between the 2 and 6 cm marks (4 cm^2). The strips were air-dried for 1 min, and individual ticks confined in a moated Petri dish were touched with an untreated end of the paper strip and allowed to mount. The paper strip was suspended vertically from a bulldog clip positioned 20 cm from a light (275 W) that shone from behind the strip, thus allowing a tick on either side of the paper to be monitored. Ticks were scored for their latencies to cross the 2 cm (Q2), 3 cm (Q3), and

6 cm marks (Q6) on the strip; the number of times they backcrossed and recrossed the 2 cm mark (Qbc); and their latencies to drop off the strip (Qld). In our coding system, Q indicates that a variable is quantitative; all variables described above are quantitative (see Kramer et al., 2009 for details). Most quantitative variables are associated with an indicator variable, 1 if the behavior was performed, and 0 otherwise (in our coding system 'Q' is replaced by 'I' to denote these indicator variables). Sessions were concluded when ticks passed the 6 cm mark, when they dropped from the paper strip or after 10 min had elapsed.

Righting and Climbing (R/C) Test We investigated toxicity by examining the R/C performances of ticks, including their ability to access a human finger, after being confined for 1 h with lemon peel exudate or test compounds (Carroll et al., 2005). A 5×6 cm rectangle of filter paper (Whatman No. 4) was marked with a lead pencil 0.5 cm from the edge. Using a pipettor, 165 μl of a test solution or acetone were applied evenly within the 4×5 cm rectangle bordered by pencil lines. After allowing the paper to air-dry for 10–15 min, it was folded crosswise, and a bulldog clip was attached to each of two margins. Eight ticks were dumped from a centrifuge tube (4 mm inner diam., truncated to 3.5 cm long, inner side coated with Fluon™) into the cavity formed by folding the paper, and another bulldog clip was attached to the open end of the packet to enclose the ticks. The packets were kept for 1 h in a glass desiccator containing water below the shelf to maintain humidity (>95%).

Immediately after a packet was opened, each nymph was placed on its dorsum on a clay substrate, which supported a vertical filter paper cylinder (diam. 3.2 cm; height 3 cm) embedded 3 mm in the clay and encircling the tick. The latencies for each tick to right itself (Qr) and climb to the top of the cylinder (Qt) were recorded. Test sessions ended after 15 min, whereupon the location of each tick (Qloc) on the bottom (recorded as 0), lower side (0.33), upper side (0.67), or on rim or outside the cylinder (1) was noted.

Following these motor performance tests, the ability of ticks to access a host was examined. One of us (J.C.) positioned the tip of his left forefinger 1–2 mm from the anterior of a (motionless or crawling) tick at 45° to its right. The tick was allowed 5 sec to climb onto the fingertip. If it failed to do so, the procedure was repeated with the right finger positioned 45° to the tick's left. If the tick still failed to climb onto the fingertip, the left and right forefingers were again proffered. Each tick had a final opportunity to climb onto the experimenter's left finger, which was placed directly in front of the tick. We scored whether a tick climbed onto a finger (Ih) and the number of trials until a tick climbed onto a finger (Qth).

Morbidity and Mortality Immediately after the R/C test, ticks were placed into a plastic vial and returned to their standard maintenance regime. At 24 h after exposure in the filter paper packet, ticks were observed in a moated Petri dish to assess survivorship. Ticks consistently moving in an uncoordinated manner and a distance of <5 mm were considered moribund. Ticks that failed to move after 5 min in the Petri dish, even when exhaled upon or prodded with forceps, were considered dead. In our analysis, we combined dead and moribund ticks because neither category poses a risk to hosts.

Statistical Methods Data were analyzed as described by Kramer et al. (2009). The basic idea was to use the behavioral differences observed as animals are tested on different compounds to find optimal weightings of these behaviors (i.e., that best discriminate among the compounds) using canonical discriminant analysis. Compounds where animals behave similarly will produce similar composite scores, whereas those compounds that induce different behaviors will produce different scores.

Time variables were square-root transformed. In addition to variables that measure duration or counts of behaviors, indicator variables were created with a value of 1 if the behavior was performed, and 0 if not. This was done so that all variables could be included in the analysis, even if not performed by all individuals. A composite score was created for individual ticks (or, for mosquitoes, a group of five mosquitoes in the chamber, described below). Useful variables to create the score were determined in a stepwise discriminant selection procedure. The composite scores we created used only the first of these canonical discriminant functions, which consisted of the sum of these variables with weights (referred to as 'loadings') from the first axis that best separated the compounds. Because the composite scores for each compound were not normally distributed, we used the non-parametric *a posteriori* Kruskal-Wallis procedure to determine which compounds differed from the acetone control and from each other. We calculated the median score and 16 and 84% quantiles for each compound. This calculation gives 68% percent coverage, so, had the data been normally distributed, these quantiles would be approximately one standard deviation on each side of the mean.

The results of the lemon, kale, and blank tests were included in the data when creating the composite score because we wanted these tests to be on the same scale as that used for the citrus compound scores, but they were analyzed separately for significant differences (i.e., after the composite scores were created, the data were split into two subsets for the Kruskal-Wallis significance tests). The reason for this is that the controls for the two subsets differed; for the lemon exudate, it was kale and blank; for the citrus compounds, it was acetone.

Two sets of analyses were conducted. One set of tick results measured avoidance, i.e., failure to enter and remain in a chemically treated zone. The other measured toxicosis, as reflected in impaired motor performance/host acquisition. The Pearson correlation between these two sets of results was calculated.

Bioassays with Mosquitoes

Membrane-Feeding Test We investigated landing and feeding responses of mosquitoes to chemically treated, artificial feeding membranes, as generally described by Weldon et al. (2006). The test apparatus was a two-piece Plexiglas module. The bottom piece was a 40×7×4 cm hollow platform supporting six circular wells (diam. 3.8 cm, depth 6 mm). Water (40°C) flowed through the central cavity of the platform. The top module piece consisted of six 4.5×4.0×5.0 cm chambers, each with a sliding floor positioned over a circular opening (diam. 3.5 cm).

The wells of the bottom piece were filled with 7 ml of a 10% sucrose solution containing ATP (2.9 mg/ml) to which 75 µl of green dye were added. In an experiment of the responses of mosquitoes to lemon peel exudate (previously reported in Weldon and Carroll 2007), a lacerated peel was pressed against a nylon-reinforced silicone membrane (0.1 mm-thick); kale-treated and blank membranes served as controls. Membranes were placed over the wells. In an experiment with individual compounds, 50 µl of a monoterpene solution or acetone were applied to membranes.

For each test session, five mosquitoes were aspirated into each of 2 or 3 chambers (depending upon the experiment) of the top module piece. The sliding floors of the chambers then were opened, allowing mosquitoes access to the membranes. The number of mosquitoes landing on the membranes, and those flying within the apparatus, were recorded each minute for 5 min. After 5 min, any mosquitoes remaining on the membranes were prodded off with a metal wire, and the sliding floor of each chamber was closed. The top piece of the module was placed into a freezer for 20 min. Mosquitoes were removed from the frozen module, crushed on white paper towels, and examined for the presence of green dye, which indicated that they had fed during a test.

Olfactometer Test We investigated the responses by mosquitoes to citrus volatiles when presented concurrently with an artificial attractant. A stock attractant solution was prepared by dissolving 0.6 g of L-(+)-lactic acid and 100 µl dimethyl disulfide in 250 ml of acetone (Bernier et al., 2007b). Two aliquots of 500 µl of this solution were pipetted into two 1.4 ml plastic vial caps (15 mm i.d. x 9.5 mm height). A 50 µl aliquot of each test compound was delivered by pipet into a smaller vial cap (9 mm i.d.×9 mm height, total capacity 400 µl). Each cap containing the attractant blend was placed on separate aluminum trays (7 x 13 cm) constructed to hold the vials. The vial cap containing the test compound was added to one of the trays.

Mosquitoes were tested in a triple-cage dual-port olfactometer to assess the relative attraction or attraction-inhibition, i.e., "repellency" (Posey et al., 1998). Each cage of the three stacked cages consists of a single unit in which a trial was conducted. In each trial, mosquitoes could remain in the cage or fly into one of the two ports containing the treatments. The test compounds were examined in a competitive bioassay, where two treatment combinations were compared (attractant blend vs. attractant blend+candidate repellent); a "blank" or "control" conditions were not used in this case.

Air for the olfactometer was drawn from outside the laboratory, and filtered, cooled, heated, and humidified or dehumidified to achieve the desired conditions for air temperature and humidity. For experiments, the air flow was 28±1 cm/sec with a temperature of 27±1°C and 60±5% RH. Approximately 75 mosquitoes were transferred to each test cage from a trap after preferential selection of host-seeking females by using a hand-draw box. Mosquitoes were loaded and allowed to acclimate in each cage of the olfactometer for 45–60 min prior to each test. During this period, the port doors were opened slightly to allow a low flow of air to pass through the ports and into the cages.

Treatment order, time of day, and ports and cages used were randomized for these trials. A total of six replicate trials was made for each treatment combination, such that each combination order was tested once in each of the two ports for each of the three cages. At the initiation of a trial, the door was opened to allow mosquitoes access to both ports of a cage. Mosquitoes trapped in each of the ports and those remaining in the cage were counted after each 3-min bioassay.

Statistical Methods For the membrane-feeding tests, data were analyzed using the methods described by Kramer et al. (2009). There were three variables collected from each five-mosquito chamber used to create the composite score. The flying counts (variable 1) were summed [total possible was 25=5 mosquitoes x 5 min (tallied each minute)] and the total count was divided by 25, with the resulting proportion arcsine transformed. The number of mosquitoes landing on the membrane (variable 2) were handled the same way. The number of mosquitoes landing elsewhere in the module at each minute was not used (to preserve independence, since the sum of the three tallies would always yield 25). The proportion of feeding mosquitoes (variable 3) also was arcsine transformed (6 possible proportions). The resulting composite scores (one score per module unit of 5 mosquitoes) were tested for compound

differences using the *a posteriori* Kruskal-Wallis method, and medians and 16 and 84% quantiles were calculated.

For the olfactometer tests, mosquitoes at the 3-min tally were either at one of the two ports or elsewhere in the cage. Thus, the data are multinomial in nature (counts in three categories). These multinomial counts could be affected by several variables, such as which treatment was present in which port, and environmental factors, such as temperature (some of these are rolled into a proxy "day" effect). Preliminary analysis of the data revealed a "day" effect for the number of mosquitoes that did not enter either port. Much of the variability in the proportions of mosquitoes entering one of two ports, based on the total number released into the olfactometer for that trial, could be removed by using instead a proportion based on only those mosquitoes that entered a port rather the total released; this is the proportion used (on the arcsine transformed scale) in the analysis. There also was a significant overall bias toward one of the ports; this was adjusted statistically by estimating the bias and subtracting its effect. Other potential variables did not appear to affect the proportions of interest. To be consistent with the other analyses, we used the Kruskal-Wallis method for determining differences among compounds, based on adjusted arcsine-transformed proportions, with the denominator of each proportion being the sum of the counts of mosquitoes at the two ports (typically between 50 and 80).

Results

Ticks The loadings of individual tick behaviors used in constructing the composite scores, and the mean separations for avoidance and the R/C test data, using all compounds are given in Table 1.

For the avoidance test, nine behaviors were deemed important for constructing the discriminant function (Table 1); the one dimension used captured 83% of the explainable variation. The most salient behavior contributing to the composite score was recrossings (Ibc, partial R^2=0.70); the next two were drop-offs (Ild, partial R^2=0.27) and latency to cross the 3 cm mark (Q3, partial R^2=0.24). Most compounds could be placed into one of two groups, those with low mean scores that were similar to the acetone control, and those with high scores, e.g., geraniol (Fig. 2). A few compounds had intermediate scores, e.g., citronellal and linalool. The axis appears to order compounds by their repellency, as desired.

For the R/C test, six behaviors were used to construct the discriminant function (Table 1). The two most important behaviors were location at end of the session (Qloc, partial R^2=0.30) and righting (Ir, partial R^2=0.20). We used only the first dimension for this score, which captured 57% of the explainable variation (the second axis, which captured about 26% of the explainable variation, separated carveol from all compounds except ß-pinene, but carveol already is well separated in the first axis) (Fig. 3). The compounds overlap considerably on the first axis, from low scores, such as those for ß-pinene and acetone, to those for the most effective compounds, such as geraniol and carveol. Only carveol impaired righting; only one tick righted after exposure to this compound. We interpret this axis to represent the differential effects of the compounds on motor performance.

Most compounds avoided by ticks also had high composite scores on the R/C test (indicating impairment),

Table 1 Loadings for tick behaviors used in constructing composite scores for repellent and righting/climbing bioassays. '*Q*' indicates that the variable is quantitative, '*I*' that it is indicator (0 or 1)

Variable Repellent Bioassay (Code)	Loading
yes/no passed the 2 cm mark (I2)	−3.616
latency to pass the 2 cm mark (Q2)	0.198
yes/no recrossed 2 cm mark (Ibc)	2.302
number recrossings of 2 cm mark (Qbc)	0.461
yes/no passed the 3 cm mark (I3)	−1.286
latency to pass the 3 cm mark (Q3)	0.128
yes/no dropped off filter paper (Ild)	2.356
latency to drop off filter paper (Qld)	−0.099
yes/no passed the 6 cm mark (Ib)	−0.654
Variable Righting/Climbing (Code)	
location (base, upper or lower side, rim) at 15 min (Qloc)	1.161
yes/no righted itself (Ir)	0.102
latency to climb to rim (Qt)	−0.001
yes/no climbed to rim (It)	1.029
yes/no climbed onto fingertip (Ih)	1.316
number of host acquisition attempts (Qth)	−0.251

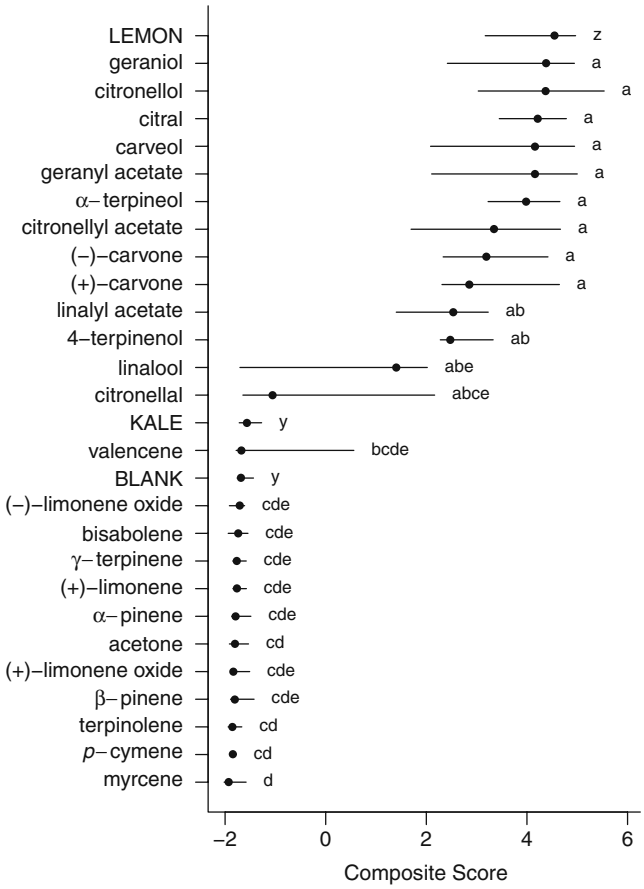


Fig. 2 Rankings of citrus compounds by composite score of five behaviors of lone star ticks to the treated portion of filter paper strips. The median scores and 16% and 84% quantiles (horizontal bars, approximately ± 1 SD) are shown. Compounds with the highest scores most repelled ticks. Note lemon and kale are exudates. Compounds with the same letters (following the horizontal bars) do not differ significantly in their effects on tick behavior. There is a separate set of letters (y and z) for plant exudates and blank

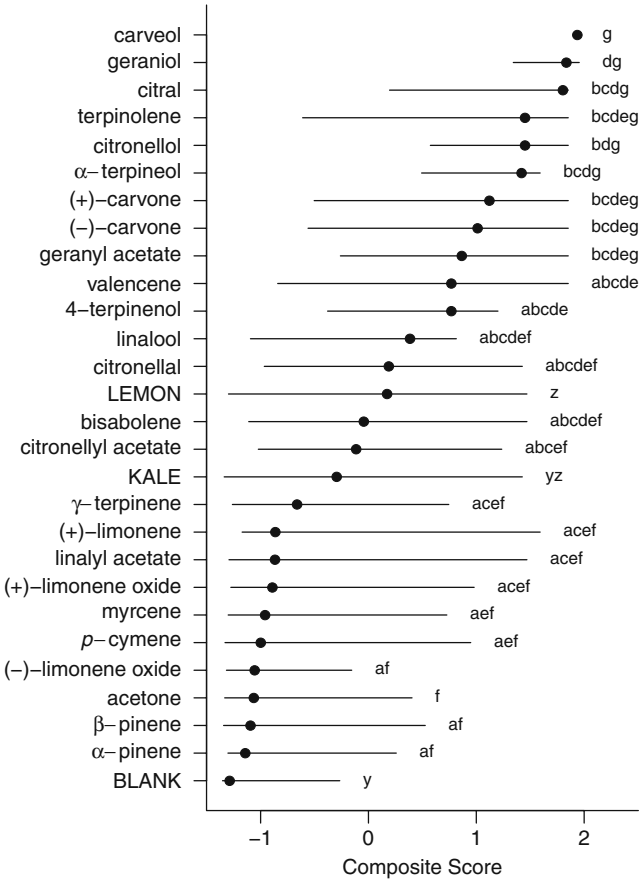


Fig. 3 Rankings of citrus compounds by composite score using righting, climbing, and crawling onto finger by lone star ticks following 1 h confinement in filter paper packet treated with test solution. The median scores and 16% and 84% quantiles (horizontal bars, approximately ± 1 SD) are shown. Compounds with the highest scores most impaired ticks. Note lemon and kale are exudates. Compounds with the same letters (following the horizontal bars) do not differ significantly. There is a separate set of letters (y and z) for plant exudates and blank

as depicted in the scatter plot in Fig. 4; the correlation coefficient of the two sets of composite scores is 0.72 ($P<0.01$). Linalool, linalyl acetate, and citronellyl acetate were avoided but did not impair behavior. Two compounds, terpinolene and valencene, appeared to impair R/C behaviors, but were not avoided. Terpinolene was the only compound for which the impairment of ticks was manifest 24 h after exposure, with 75% of subjects moribund and 17% dead.

Mosquitoes The responses by mosquitoes to nine of the compounds in the membrane-feeding tests were statistically indistinguishable from those to the acetone control. The others had composite scores that differed from the acetone control; 12 compounds/isomers had similar high scores (these are followed by "d" or "de" in Fig. 5). All of the alcohols occurred in this group, as did both isomers of limonene oxide.

In the olfactometer test (Fig. 6), responses only to linalool ("repellent") and two compounds, citronellal and γ-terpinene ("attractants"), differed significantly ($P<0.05$) from each other. Most of the compounds had at least one trial proportion on each side of the 50% (inactive) line, and did not differ ($P>0.05$) from other compounds.

Results from the membrane-feeding test and olfactometer test had essentially no relationship to each other (Fig. 7, Pearson correlation=−0.219, $P=0.28$).

Discussion

Lone star ticks in our study avoided lemon peel exudate and, after confinement with it, exhibited impaired climbing behavior. These responses are consistent with the deterrence of ticks by citrus-derived chemicals applied via anointing, as are our results with a number of compounds we tested.

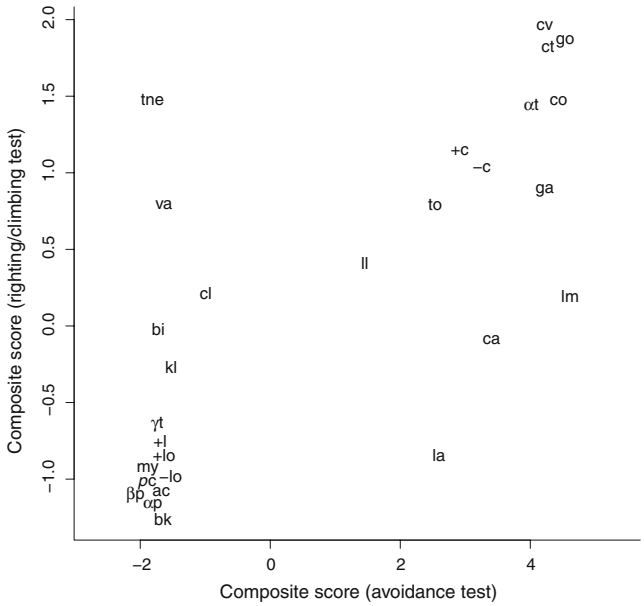


Fig. 4 Scatterplot of composite scores from avoidance (repellent) test and from righting/climbing (motor impairment) test for lone star ticks. Compounds tested were *p*-cymene (*p*c), bisabolene (bi), (+)-limonene (+l), myrcene (my), α-pinene (αp), ß-pinene (βp), γ-terpinene (γt), terpinolene (tne), valencene (va), citral (ct), citronellal (cl), (−)-carveol (cv), citronellol (co), geraniol (go), linalool (ll), 4-terpinenol (to), α-terpineol (αt), (+)- carvone (+c), (−)-carvone (−c), (+)- limonene oxide (+lo), (−)-limonene oxide (−lo), citronellyl acetate (ca), geranyl acetate (ga), and linalyl acetate (la). Most compounds displayed similar rankings in the two tests, with some exceptions (e.g., terpinolene, "tne", impaired motor performance but was not avoided). Note lemon (lm) and kale (kl) are exudates. Compound codes that overlapped were moved slightly apart for readability

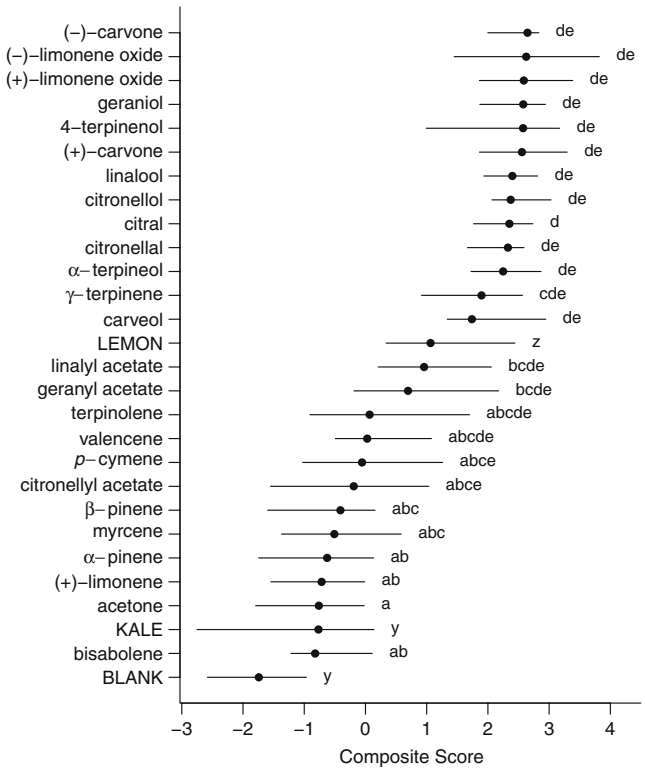


Fig. 5 Rankings of citrus compounds by composite score, using flying, landing, and feeding by yellow fever mosquitoes confined in module containing sucrose solution covered by a membrane treated with test solution. The median scores and 16% and 84% quantiles (horizontal bars, approximately ± 1 SD) are shown. Compounds with the highest scores most inhibited landing and feeding, and elicited flying. Note lemon and kale are exudates. Compounds with the same letters (following the horizontal bars) do not differ significantly in their effects on mosquito behavior. There is a separate set of letters (y and z) for plant exudates and blank

Previous studies on the responses by ectoparasitic acarines to the monoterpenes we examined focus chiefly on mortality. Four monoterpenes tested against the ear mite (*Otodectes cynotis*) exhibited the following order of miticidal potency: geraniol > limonene > *p*-cymene > α-pinene (Traina et al., 2005). A structure-activity study of the contact and fumigation toxicities of 13 monoterpenes against the mange mite (*Psoroptes cuniculi*) indicated mortalities at or near 100% for most doses of geraniol, linalool, α-terpineol, and 4-terpinenol, but failed to indicate limonene, myrcene, or γ-terpinene as toxic; linalyl acetate exhibited intermediate toxicity (Perrucci et al., 1995). The observation of Perrucci et al. (1995) that alcohols are the best miticides against mange mites, and that hydrocarbons are least effective, is consistent with our toxicity results with lone star ticks, where carveol was the most toxic compound, and α- and β-pinene were among the least toxic. Interestingly, a study of the mortality of the southern cattle tick (*Rhipicephalus* [*Boophilus*] *microplus*) to monoterpenes indicated the following order of efficacy: citronellol > α-pinene = β-pinene > linalool (Prates et al., 1998).

The monoterpenes we tested with the lone star tick have been tested as repellents with other tick species. Valencene and 4-terpinenol repelled the blacklegged tick (*Ixodes scapularis*), but not as effectively as did some other terpenes examined (Dietrich et al., 2006). Citronellol, geraniol, α-terpinenol, 4-terpinenol, and α-pinene repelled nymphs of the sheep tick (*Ixodes ricinus*) (Thorsell et al., 2006; Tunón et al., 2006; Pälsson et al., 2008), but linalool was ineffective (Tunón et al., 2006; Del Fabbro and Nazzi, 2008). The cattle tick (*Rhipicephalus appendiculatus*) also was repelled by geraniol and α-terpineol, and, to some extent, myrcene (Ndungu et al., 1995; Lwande et al., 1999). In our study, alcohols—geraniol, citronellol, carveol, and α-terpineol—were among the most potent repellents (see also Kramer et al., 2009). Citral, geranyl acetate, citronellyl acetate, and carvone also were strongly avoided. On the other hand, hydrocarbons, including myrcene and *p*-cymene, were least repellent. Thus, the compounds that most repel our ticks also are most toxic to them, although exceptions

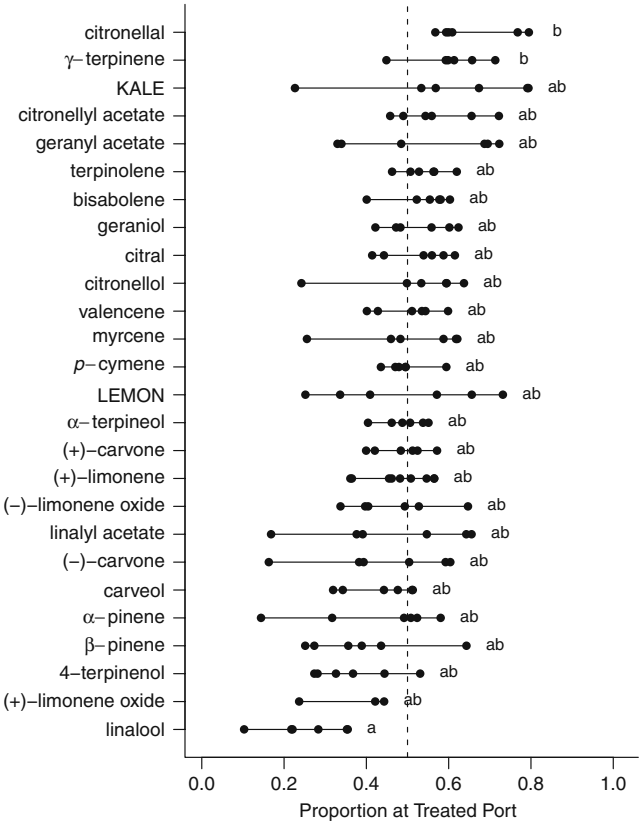


Fig. 6 Proportion of yellow fever mosquitoes at olfactometer port with test compound + attractant blend. Mosquitoes could enter the port with attractant blend only, the port with attractant blend + test compound or remain elsewhere in the olfactometer. The proportions shown are based on only numbers of mosquitoes at the ports at 3 min. Each circle represents the results from one trial. For ease of interpretation, a line connects the results of each of the trials of a compound. Note lemon and kale are exudates. Compounds with the same letters (following the horizontal bars) do not differ significantly in their effects on mosquito behavior. A central dotted line at 0.5 indicates an equal tendency to enter either port. Points further from the line denote greater attractiveness (*right*) or repellency (*left*) of the compounds

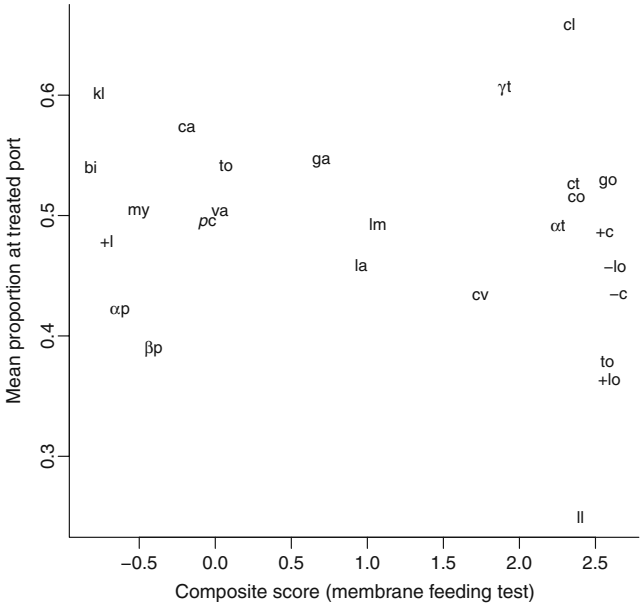


Fig. 7 Scatterplot of composite scores of yellow fever mosquitoes from membrane-feeding test and mean proportion at treatment port from olfactometer test. Results for the two tests appear unrelated. Compounds abbreviated as in Fig. 4. Note lemon and kale are exudates. Compound codes that overlapped were moved slightly apart for readability

occur, e.g., terpinolene, which was not avoided but did impair motor performance.

Mosquitoes in our module tests landed and fed least, and flew most, when confined with lemon peel exudate versus kale and blank controls. These results were interpreted to reflect repellence by lemon peel volatiles (Weldon and Carroll, 2007). In our olfactometer, however, lemon peel exudate did not appear to affect mosquito behavior when the insects were presented concurrently with the artificial attractant solution. Moreover, the application of fresh lemon peel exudate to the arm of a human subject (P.J.W.) failed to deter biting by yellow fever mosquitoes. Buckley (1983), who reported that white-faced capuchin monkeys in Honduras fur-rub with oranges and grapefruit, also failed to sustain fewer bites by deer flies (*Chrysops* sp., Tabanidae) when he applied orange peel exudate to himself.

Some of the monoterpenes we tested, such as *p*-cymene (Choi et al., 2002; Park et al., 2005; Waliwitiya et al., 2009), carvone (Vartak and Sharma, 1993), citral (Hao et al., 2008), citronellal (Waliwitiya et al., 2009), geraniol (Hao et al., 2008; Müller et al., 2009), terpineol (Traboulsi et al., 2005), and linalool (Choi et al., 2002; Park et al., 2005), have been reported to repel adults of some mosquitoes. Tests of yellow fever mosquitoes often have indicated the greater repellency of alcohols, aldehydes, and related compounds compared to hydrocarbons (Hwang et al., 1985; Zaki et al., 2005), thus supporting Bunker and Hirschfelder's (1925) early observation on the greater overall efficacy of oxygenated compounds as mosquito repellents. Our olfactometer results confirmed Kline et al.'s (2003) result that linalool strongly repels yellow fever mosquitoes, but the olfactometer test did not discriminate among compounds as well as did the module assay; there was no correlation between the results of these two assays.

Higher concentrations of volatiles undoubtedly were presented to mosquitoes in the module than in the olfactometer, both in tests with lemon peel exudate and individual compounds. Some of these volatiles, even those that in low doses attract mosquitoes, may repel them at high concentrations (Bernier et al., 2007a; Butler, 2007). Aside from the likely concentration differences of volatiles in the assay systems, the disparity in the results of our mosquito assays may be due to the kind of data collected and how

they were analyzed. The olfactometer test scored only one binary behavior variable, and this behavior is related to anemotaxis toward an attractive odor source; the membrane system measured close-range deterrence, which may reflect "repellency." Moreover, the membrane-feeding test scored three behaviors per unit (five mosquitoes), then combined them into a composite score by a weighted summation, with weights to produce scores that separate the compounds; this more sensitive test demonstrated that mosquitoes discriminated among our compounds.

More fundamentally, it is unclear that the module assay applied to mosquitoes distinguishes between compounds that deter landing (as repellents) and those that elicit sustained flight, as by triggering upwind attraction. To clarify this issue, a range of compounds that have well characterized behavioral effects on mosquitoes needs to be tested in assays that distinguish between repellence and flight stimulation.

Some authors focus on limonene in citrus and other plant extracts as a prime deterrent of ticks (Chungsamarnyart and Jansawan, 1996), mosquitoes (Gillij et al., 2008), or arthropods in general (Ibrahim et al., 2001). Limonene in our study did not emerge as a potent deterrent of either ticks or mosquitoes. Nonetheless, limonene in citrus fruits may contribute to deterring some arthropods by virtue of its abundance; it comprises >90% of the volatiles of some citrus extracts (Dugo et al., 2002). In addition, limonene applied to the integument of anointed animals may be oxidized on the high surface area of the pelage or plumage into more active agents, such as carveol (Karlberg et al., 1992), which was highly repellent and toxic to our ticks. Similar chemical transformations may occur for other components present in citrus and other anointing materials, thus rendering deterrents from ineffective compounds.

Conjecture on the importance of vertebrate anointing as a defense against hematophagous arthropods should consider the availability of anointing materials in nature. *Citrus* spp. originated in Southeast Asia and were transported to America by Portuguese and Spanish conquistadores during the early 1500s (Calabrese, 2002). Citrus trees now are widespread in the New World, but they occur chiefly in cultivated regions, thus they may not be encountered routinely by free-ranging animals. For example, white-faced capuchin monkeys in a region of Honduras devoid of citrus trees were observed to fur-rub only with fruits acquired from humans (Buckley, 1983). The monoterpenes we examined may occur in other materials used for anointing. It is worth noting that some ants, including those used by birds in anointing, such as *Acanthomyops* spp. and *Lasius* spp. (Whitaker, 1957), defensively discharge citral, citronellal, citronellol, and related compounds (Blum, 1981).

Falótico et al. (2007) suggested that tufted capuchin monkeys (*Cebus apella*) anoint with ants in order to acquire formic acid and other topical deterrents of tick nymphs. Similary, DeJoseph et al. (2002) suggested that white-faced capuchin monkeys anoint with plants, including citrus fruits, to combat lice, mites, and ticks. Ticks may be especially vulnerable to topically applied chemicals because they engage in protracted feeding on the integument of hosts (Carroll et al., 2005).

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RAPID COMMUNICATION

The Biosynthesis of Hexahydrofarnesylacetone in the Butterfly *Pieris brassicae*

Stefan Schulz · Selma Yildizhan · Joop J. A. van Loon

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Abstract Hexahydrofarnesylacetone (6,10,14-trimethylpentadecan-2-one, **1**) is a widespread ketone occurring in plants and insects. Several species use this compound or the respective alcohol as part of their pheromone bouquet. Here, we showed by using deuterium labeled phytol (**3**) and GC-MS experiments that the Large Cabbage White butterfly *Pieris brassicae* can take up phytol in the larval stage and transforms it into **1** by oxidative degradation.

Key Words Biosynthesis · Pheromone · Labeled compounds · Terpenes

Introduction

Hexahydrofarnesylacetone (6,10,14-trimethylpentadecan-2-one, phytone, HHFA, **1**), is a relatively common natural product, occurring in many plants, often as part of the floral odor bouquet, and in several insect species (Schulz et al., 1993; Schulz and Nishida, 1996; Vendilo et al., 1998; Sasaerila et al., 2003; Kalinova et al., 2009). Closely related is the corresponding alcohol, 6,10,14-trimethylpentadecan-2-ol that occurs in the pheromone gland of several Lepidoptera (Mori et al., 1991; Sasaerila et al., 2003; Nieberding et al., 2008). Recently, Eltz et al. (2010) showed that Orchid bees are attracted to the (*R*,*R*)-enantiomer of **1**, produced by many orchids, and store this compound in their tibial fragrance. These authors also established the absolute configuration of insect-derived **1** for the first time. We have found that *Pieris* butterflies use **1** as a component of the aphrodisiac pheromone of the males (Yildizhan et al., 2009). Hexahydrofarnesylacetone is a known degradation product of (*E*)-phytol (**3**), a diterpene alcohol that occurs as a side chain of chlorophyll a in all plants. This degradation proceeds abiotically by oxidative processes. Nevertheless, the biogenic formation of **1** has not been studied before. Different plausible biosynthetic routes exist to this ketone. An enzymatic oxidative cleavage of **3** could lead to **1**. Alternatively, oxidative degradation of the tetraterpene and carotenoid precursor lycopersene or related compounds, and reduction of the respective double bonds also would lead to **1**. Finally, degradation of tocopherol, which is produced by green plants and commonly found in plant feeding insects, could furnish **1** in the end. In this communication, we show that **1** is indeed directly derived from **3,** and is synthesized by the Large Cabbage White, *Pieris brassicae*.

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Methods and Materials

Analytical Methods GC/MS analyses were performed as follows: an HP 6890 GC was coupled with an HP 5973 mass selective detector, splitless injection, BPX-5 column (SGE), 60–320°C, 5°C/min. In nuclear magnetic resonance (NMR) experiments, a DRX-400 (400 MHz, Bruker) instrument was used.

Synthesis of Compounds A 3:1 mixture of D-labeled (*E*)- and (*Z*)-**3** was obtained from **1** (Fig. 1a). An H/D exchange

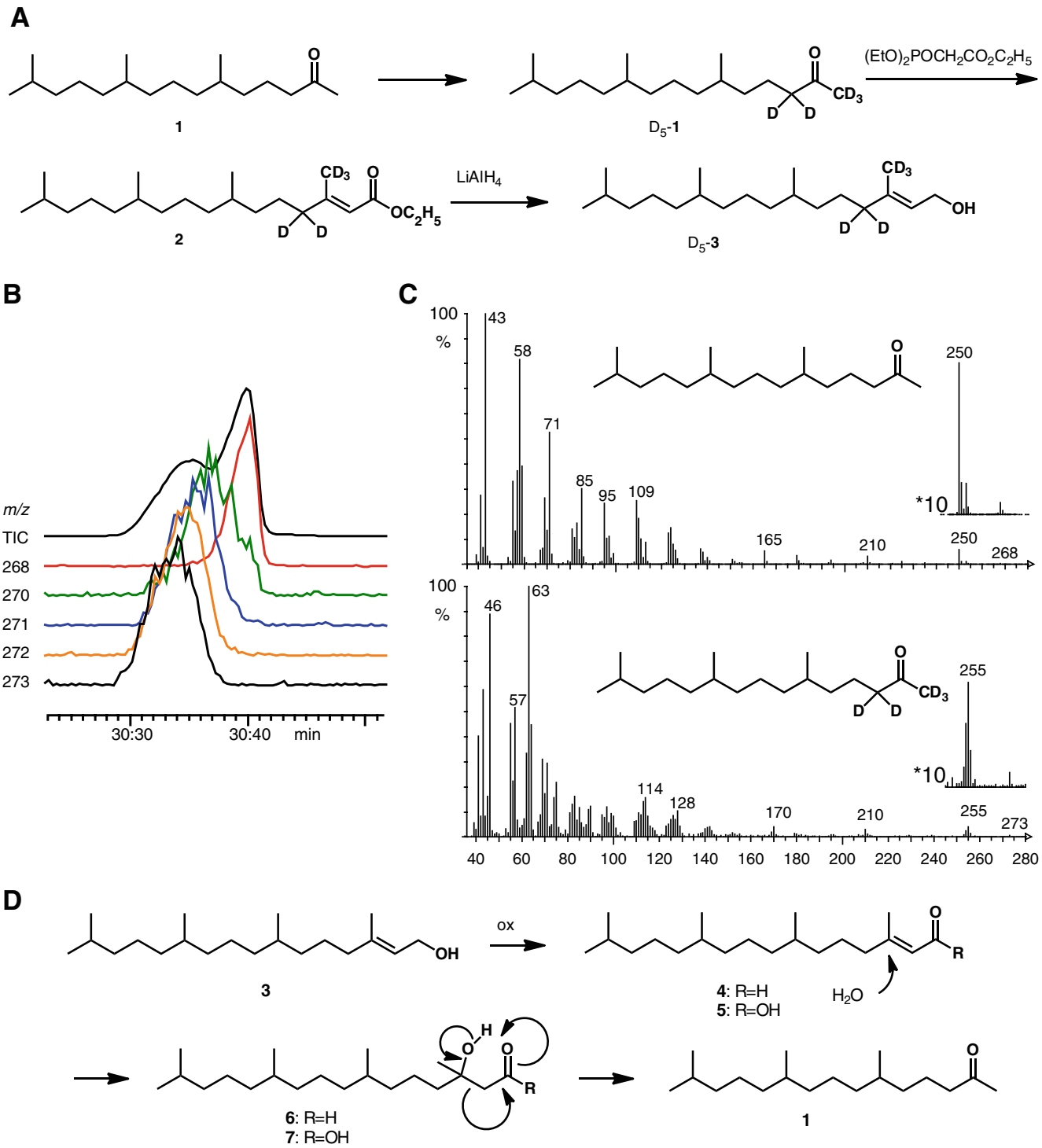


Fig. 1 **A**: Synthesis of deuterated phytol. **B**: Total ion chromatogram with ion traces of molecular ions of the different isotopomers of **1** from an extract of wings of male *Pieris brassicae* after feeding the larvae with D_5-**3**. **C**: Mass spectra of **1** and D_5-**1**, obtained in the feeding experiments. **D**: Proposed biosynthetic pathway from **3** to **1** in *P. brassicae*

at the α-positions next to the carbonyl group was performed under basic conditions with NaH/CH_3OD. The thus obtained 1,1,1,3,3-D_5-**1** was transformed into a mixture of ethyl (*E*)- and (*Z*)-phytoenoate (**2**) by a standard Horner-Wadsworth-Emmons reaction with triethyl phosphonoacetate. This ester was reduced finally with $LiAlH_4$ to give D_5-phytol (**2**), and purified by column chromatography. The analysis of the product revealed that the D-exchange was not complete, but sufficient for our experiments. The ratio of the different isotopomers D_0:D_1:D_2:D_3:

D_4:D_5 was 4:4:8:22:35:27. D-**3**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.34 (t, 1H, H-2), 4.08 (d, 0.75H, *E*-H-1), 4.05 (d, 0.25H, *Z*-H-1), 1.0–1.6 (m, 22H), 0.79 (d, 6H), 0.78 (d, 3H), 0.76 (d, 3H).

Feeding Experiments Pieris brassicae fifth instar caterpillars were obtained from a laboratory colony reared on greenhouse-grown *Brassica oleracea* L. var. *gemmifera* cv. Cyrus (Sluijs & Groot, Enkhuizen, The Netherlands), in climate controlled rooms at 22°C ±2°C, 50% RH, 16:8 hL:D. Caterpillars were put singly into Petri dishes when they had reached a body weight of ca. 450 mg, as this indicates that feeding will cease within a few hours to be followed by the wandering phase and formation of the prepupa. Individuals were offered a leaf disk of *B. oleracea* on which a solution of labeled phytol in 5% ethanol in water was surface-applied (1 mg phytol per leaf disk). After the leaf disk was fully consumed, caterpillars were deprived of food and kept in the dish until pupation. Pupae were sexed and male pupae were mounted in a cage for eclosion. Males were kept for 5 d in a cage provided with a 10% solution of sucrose in tap water, after which their wings were dissected and immediately extracted with 100 L dichloromethane for 10 min using stainless steel forceps.

Results

Deuterium-labeled phytol (**3**) was synthesized starting from **1** by H/D-exchange under basic conditions. By this approach, the label is in positions that will not be affected by the proposed transformation of phytol. The labeled **3** then was fed to *P. brassicae* larvae, and the wings of the resultant adult males were extracted. Analysis of the extract by GC-MS showed the presence of deuterium labeled isotopomers of **3** in a roughly 1:1 ratio to the unlabeled compound (Fig. 1). Particulary instructive is the mass spectrum of the earliest eluting isotopomer with the molecular ion at *m/z* 273, indicating the retention of all five D-atoms of the labeled phytol. The ion shifts from *m/z* 43 to 46 and *m/z* 58 to 63 locate the D-atoms in the positions C-1 and C-3, because they are formed either by α-cleavage next to the carbonyl group or by McLafferty-rearrangement typical for methylketones. The degradation of the double bond would lead to this labeling pattern. All D-atoms found in the labeled phytol are present in the product **1** in identical isotopomer distribution.

Discussion

Chlorophyll a is taken up by most chewing insects during feeding on plants, and is degraded during digestion, releasing (*E*)-phytol (**3**) (Matile et al., 1999). The larvae of *P. rapae* is thus taking up **3** and accumulating it in the scent scales of the wings of males. In the males, **3** is at least in part degraded to **1**. This is different from orchid bees that take up **1** from plants (Eltz et al., 2010). The mechanism of this cleavage is unknown, but a plausible explanation is shown in Fig. 1. Phytol can be oxidized to either phytenal (**4**) or even further to phytoenic acid (**5**). The now established α,β-unsaturated carbonyl system can act as a Michael-acceptor and add water as a nucleophile. A retro-aldol-fragmentation of the hydroxycarbonyl compounds **6** or **7** follows that releases hexahydrofarnesylacetone and acetaldehyde or acetate. The intermediate **4** can be found as a trace component in the male wing extracts (Yildizhan et al., 2009). Alternatively, a direct oxidative cleavage of the double bond of **1** is possible, but the dioxygenase enzymes capable of doing this usually act on aromatic systems. The formation of **1** by degradation of tocopherol seems less likely because it would require cleavage of a non-activated C-C bond. The formation from tetraterpenes also is not supported by our data because in that case no deuterium incorporation would be expected. It seems likely that plants synthesize **1** by a similar pathway. In all known cases, natural **1** and **3** share the same absolute (6*R*,10*R*)-configuration in the chain. The described pathway also may be active for other degraded terpenes like sulcatone or geranylacetone, derived from geraniol or farnesol, in addition to the already known formation by direct oxidation of open-chain carotenoids like lycopene (Vogel et al., 2008).

In essence we have shown that male *P. brassicae* synthesize hexahydrofarnesylacetone (**1**) from phytol (**3**), originating from chlorophyll a degradation. This may explain the relatively widespread occurrence of this compound in both plants and insects.

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RAPID COMMUNICATION

Risk of Egg Parasitoid Attraction Depends on Anti-aphrodisiac Titre in the Large Cabbage White Butterfly *Pieris brassicae*

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Abstract Males of a variety of insects transfer an anti-aphrodisiac pheromone to females during mating that renders them less attractive to conspecific males. In cabbage white butterflies, the transfer of an anti-aphrodisiac can result in the unwanted attraction of tiny egg parasitoid wasps of the genus *Trichogramma* that hitch-hike with mated female butterflies to a host plant where they parasitize the freshly laid butterfly eggs. Here, we show that the anti-aphrodisiac benzyl cyanide (BC) of the large cabbage white *Pieris brassicae* is depleted by frequent display of the mate-refusal posture that signals a female's unreceptivity to mating. This depletion of BC is ecologically important because it results in a reduced risk of attracting the hitch-hiking egg parasitoid *Trichogramma brassicae* to mated female butterflies over time since mating. Our results indicate for the first time that a reduction in anti-aphrodisiac titre in mated females due to frequent adoption of the mate-refusal posture is beneficial to both mated females and males particularly when parasitoid pressure is high.

Key Words Benzyl cyanide · Pheromone · Polygamy · Mate-refusal posture · Chemical espionage · *Trichogramma brassicae*

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Introduction

In various butterfly species with female polygamy, the male transfers an anti-aphrodisiac pheromone blend to the female during mating to reinforce monogamy (Andersson et al., 2000, 2003, 2004; Schulz et al., 2008). This not only benefits the male, because it reduces the risk of sperm competition, but also the recently mated female, because a reduced harassment by other males enables her to invest more in offspring production (Forsberg and Wiklund, 1989). However, the use of an anti-aphrodisiac is vulnerable to chemical espionage by natural enemies (Fatouros et al., 2005b; Huigens et al., 2009, 2010).

Males of the large cabbage white butterfly *Pieris brassicae* transfer the anti-aphrodisiac benzyl cyanide (BC) to females during mating, which renders them less attractive to conspecific males, whereas males of the green-veined white *Pieris napi* transfer methyl salicylate (MeSA) (Andersson et al., 2000, 2003, 2004). In *P. napi*, the storage of MeSA in the mated female is depleted over time after mating by frequent adoption of a posture that signals unreceptivity to mating (Andersson et al., 2004). This so called 'mate-refusal' posture with spread wings and elevated abdomen is displayed both at male and female butterflies and is typical for all Pieridae (Obara, 1964).

In this study, we examined whether depletion of an anti-aphrodisiac has ecological consequences for both male and female butterflies. Recently, we discovered how the use of the anti-aphrodisiac BC in *P. brassicae* can be exploited by the tiny egg parasitoid wasps *Trichogramma brassicae* and *T. evanescens*; after the wasps detect BC released by a female butterfly on day 1 after mating, they mount her and hitch a ride to a host plant where they parasitize her freshly laid eggs (Fatouros et al., 2005b; Huigens et al., 2009). Here, we investigated whether the amount of BC released by mated

female *P. brassicae* butterflies a) changes over time since mating, b) is influenced by frequent display of the mate-refusal posture, and c) mediates the risk of attracting the hitch-hiking egg parasitoid *T. brassicae* to mated female butterflies.

Methods and Materials

Butterflies and Wasps *Pieris brassicae* L. (Lepidoptera: Pieridae) butterflies and *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) wasps (iso-female strain Y175) were reared as described in previous studies (Fatouros et al., 2005a, b; Huigens et al., 2009). We used only mated, 2-d-old female wasps in the experiments. Singly-mated female butterflies were obtained by adding virgin males to virgin females in a cage (50×50×50 cm) 2 days after eclosion. Mating couples were individually isolated in plastic cylinders (15 cm i.d., 30 cm high) covered with a gauze lid. After a couple had separated, the male was removed, and the female was provided daily with fresh honey water (10%). On day 1 after mating, females were kept individually, and volatiles were collected for 1.5 hr (see below). After BC collection, females were either housed individually, or housed together with 6 other mated females. Benzyl cyanide was collected again on day 5 after mating (Andersson et al., 2004). All butterflies were active, but females only (frequently) displayed the mate-refusal posture when housed in a group (Andersson et al., 2004).

Emission of BC A dynamic headspace system was used to collect BC that is released by one singly-mated female butterfly. An individual female was confined in a glass jar (0.5 L, Weck, Germany) that was closed with a Viton-lined glass lid having an inlet and outlet. Inlet air was filtered by passing through a stainless steel cartridge filled with 200 mg of charcoal. Volatiles were trapped by sucking air out of the jar at a rate of 100 ml min^{-1} through a similar cartridge filled with 200 mg Tenax TA (20/35 mesh; Grace-Alltech, USA). Benzyl cyanide was collected for 1.5 hr on day 1 and day 5 after mating. To determine whether a female butterfly releases more BC when it displays the mate-refusal posture, we added a virgin [BC free (Andersson et al., 2003)] female butterfly to the glass jar during volatile trapping. Headspace samples were analyzed with a Thermo Trace GC connected to a DSQ mass spectrometer (Thermo Fisher Scientific, USA). Volatiles were thermally desorbed from Tenax cartridges at 250°C for 3 min (Ultra, Markes Llantrisant, UK) with a helium flow of 30 ml min^{-1}. Analytes were focused at 0°C on an electronically-cooled sorbent trap (Unity, Markes, Llantrisant, UK) and were then transferred in splitless mode to the analytical column (ZB-5Msi, 30 m, 0.25 mm i.d., 1.0 μm film thickness, Phenomenex, USA) by rapid heating of the cold trap to 250°C for 5 min. The temperature gradient of the GC was as follows: 60°C hold for 3 min, 10°C min^{-1} gradient to 200°C, then 40°C min^{-1} gradient to 280°C, 2 min hold, constant column flow of 1 ml min^{-1}). Mass spectra were acquired by electron impact ionization (70 eV) with a scanning from 45–200 m/z with a scan rate of 7.3 scans s^{-1}. Benzyl cyanide was identified by comparing the mass spectrum and retention time with an authentic reference standard. A linear calibration curve made from 0.1, 1, 10, 100, and 1000 ng synthetic BC was used to determine the amount of BC released by mated female butterflies.

Wasp Response to Mated Female Butterfly Odors In a static two-chamber olfactometer, we tested the response of *T. brassicae* to odors released by differently treated singly-mated female butterflies (1dI=1 day after mating, housed individually; 5dI=5 days after mating, housed individually; 5dG=5 days after mating, housed in a group) as previously described (Fatouros et al., 2005b; Huigens et al., 2009, 2010). The olfactometer differed only in height (7 cm) from the one that was used before (18 cm). One *P. brassicae* female per chamber was introduced as odor source. The time spent (residence time) by the wasps in one of the two odor fields was observed for 300 sec.

Results and Discussion

The gas chromatography-mass spectrometry (GC-MS) analysis shows that the storage of BC in mated female *P. brassicae* butterflies is depleted over time by frequent display of the mate-refusal posture. Co-habitating female butterflies released significantly less BC on day 5 than on day 1 (Fig. 1a; *Wilcoxon signed-rank test*, $P=0.003$). This is consistent with the fact that when housed in a group, females frequently adopted the male-refusal posture (Andersson et al., 2004). This posturing does not occur as frequently when a female is housed individually (Andersson et al., 2004). Therefore, BC levels emitted from individually housed females were lower than day 1 levels, although they were not statistically different (Fig. 1a; *Wilcoxon signed-rank test*, $P=0.480$). On day 5 after mating, female butterflies that were housed alone also released significantly more BC than those that were housed in a group (Fig. 1a; *Mann–Whitney U test*, $P=0.002$). Moreover, mated female butterflies that were maintained together with a virgin female during volatile trapping, and consequently exhibited the mate-refusal posture, released significantly more BC than those that were maintained alone (Fig. 1a; *Mann–Whitney U test*, $P=0.001$). The anti-aphrodisiac thus, is released mostly during the mate-refusal posture in *P. brassicae*, just as in *P. napi* (Andersson et al., 2004), thereby suggesting this phenomenon to be more widespread among Pierid butterflies.

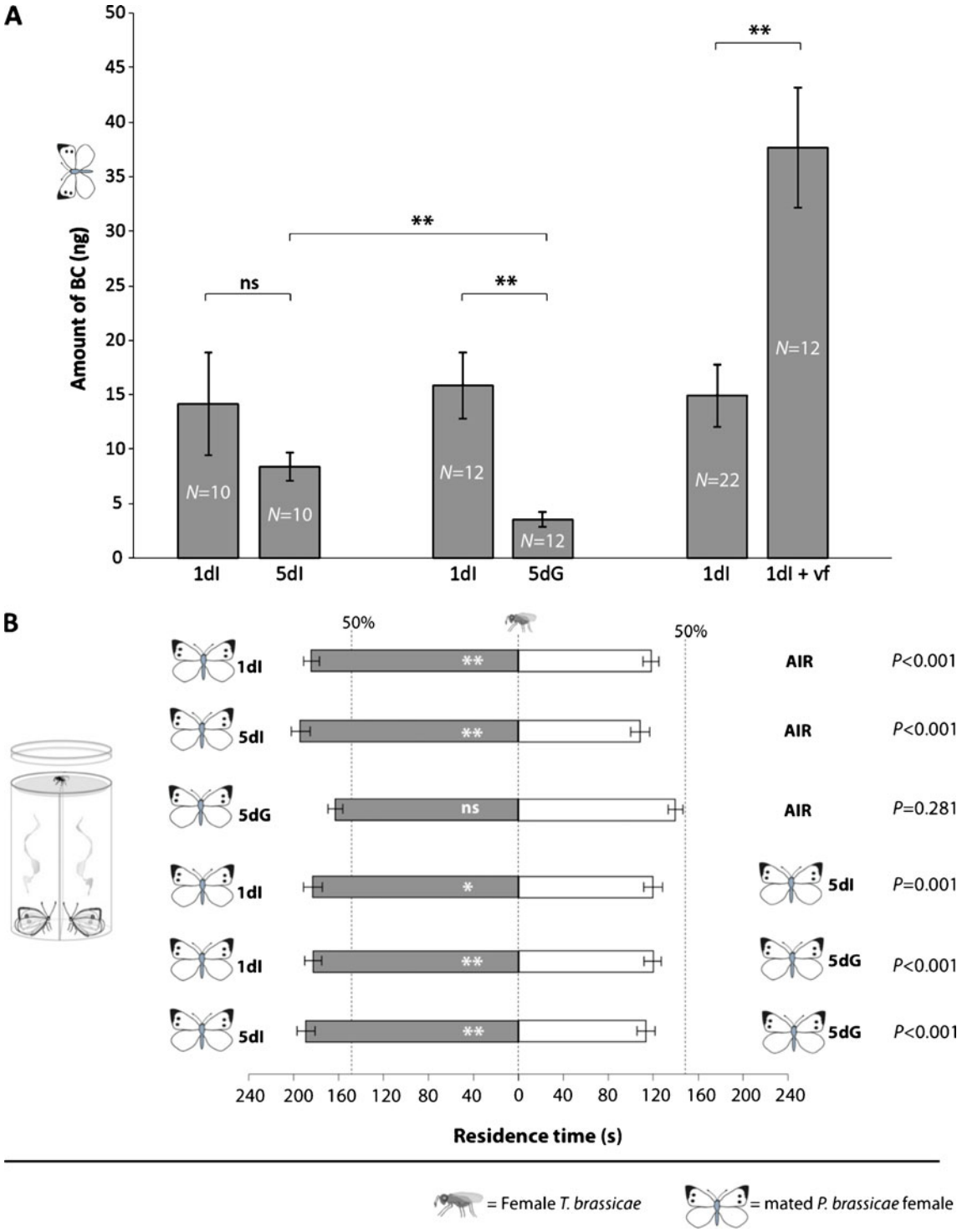


Fig. 1 **a** Amount of BC (ng) released by differently treated singly-mated females of the large cabbage white butterfly *Pieris brassicae* on day 1 and days 5 after mating during 1.5 hr of volatile trapping. **b** Response of *Trichogramma brassicae* wasps to the odors of differently treated singly-mated female butterflies on day 1 and days 5 after mating; mean residence time (± s.e.m.) in two odor fields in a static olfactometer; N=40 wasps tested per experiment. 1dI=1 day after mating, housed individually; 5dI=5 days after mating, housed individually; 5dG=5 days after mating, housed in group; 1dI + vf=1 day after mating, housed individually, accompanied by a virgin (BC free) *P. brassicae* female during volatile trapping. In **a**, a *Wilcoxon signed-rank test* was used for comparisons of 1dI with 5dI (same female butterflies) and 1dI with 5dG (same female butterflies), and a *Mann–Whitney U test* for comparisons of 5dI with 5dG (different female butterflies) and 1dI with 1dI + vf (different female butterflies). In **b**, we used a *Wilcoxon signed-rank test* for comparisons within a choice test. *Asterisks* indicate significant differences, $^{**}P<0.01$, ns: not significant

Our results indicate that a depletion of BC in female *P. brassicae* butterflies over time since mating has important ecological consequences. In two-choice olfactory bioassays, *T. brassicae* wasps showed clear odor preferences for mated *P. brassicae* females that released the largest amount of BC. When offered against clean air, odors of female butterflies that were housed alone significantly arrested the wasps on day 1 and day 5 after mating (Fig. 1b; *Wilcoxon signed-rank test*, $P<0.001$ and $P<0.001$, respectively). However, wasps did not discriminate between odors of female butterflies that were housed in a group until day 5 after mating and clean air (Fig. 1b; *Wilcoxon signed-rank test*, $P=0.281$). Wasps also were more strongly arrested by female butterfly odors 1 day after mating when tested against odors of female butterflies that were either housed alone, or in a group, until day 5 after mating (Fig. 1b; *Wilcoxon signed-rank test*, $P<0.001$ and $P<0.001$, respectively). Although the amount of BC released by 5-d-individually housed mated females is not significantly lower than that released by 1-d-individually housed mated females, it might be sufficiently lower to result in a reduced arrestment of *T. brassicae* wasps.

In nature, recently mated female *P. brassicae* butterflies are likely to run a higher risk of attracting the hitch-hiking egg parasitoid *T. brassicae* than do older mated females that have frequently adopted the mate-refusal posture, and consequently emit less BC. The mutual benefit of using an anti-aphrodisiac for both the mated female and the male (Forsberg and Wiklund, 1989; Andersson et al., 2004) could turn into a conflict over time after mating, because the female may benefit later from mating a second time, i.e., from a reduction in anti-aphrodisiac release, whereas the male does not (Andersson et al., 2004). Our results, however, suggest that a decrease in anti-aphrodisiac emission by the female due to a frequent display of the mate-refusal posture after mating also is beneficial for the male because it reduces the risk of indirectly attracting natural enemies to his offspring via the mated female.

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Differences in Volatile Profiles of Turnip Plants Subjected to Single and Dual Herbivory Above- and Belowground

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Abstract Plants attacked by herbivorous insects emit volatile organic compounds that are used by natural enemies to locate their host or prey. The composition of the blend is often complex and specific. It may vary qualitatively and quantitatively according to plant and herbivore species, thus providing specific information for carnivorous arthropods. Most studies have focused on simple interactions that involve one species per trophic level, and typically have investigated the aboveground parts of plants. These investigations need to be extended to more complex networks that involve multiple herbivory above- and belowground. A previous study examined whether the presence of the leaf herbivore *Pieris brassicae* on turnip plants (*Brassica rapa* subsp. *rapa*) influences the response of *Trybliographa rapae*, a specialist parasitoid of the root feeder *Delia radicum*. It showed that the parasitoid was not attracted by volatiles emitted by plants under simultaneous attack. Here, we analyzed differences in the herbivore induced plant volatile (HIPV) mixtures that emanate from such infested plants by using Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA). This multivariate model focuses on the differences between odor blends, and highlights the relative importance of each compound in an HIPV blend. Dual infestation resulted in several HIPVs that were present in both isolated infestation types. However, HIPVs collected from simultaneously infested plants were not the simple combination of volatiles from isolated forms of above- and belowground herbivory. Only a few specific compounds characterized the odor blend of each type of damaged plant. Indeed, some compounds were specifically induced by root herbivory (4-methyltridecane and salicylaldehyde) or shoot herbivory (methylsalicylate), whereas hexylacetate, a green leaf volatile, was specifically induced after dual herbivory. It remains to be determined whether or not these minor quantitative variations, within the background of more commonly induced odors, are involved in the reduced attraction of the root feeder's parasitoid. The mechanisms involved in the specific modification of the odor blends emitted by dual infested turnip plants are discussed in the light of interferences between biosynthetic pathways linked to plant responses to shoot or root herbivory.

Key Words Plant–insect interactions · Multiple herbivory · Volatile organic compounds · Parasitoid · *Trybliographa rapae* · *Delia radicum* · *Pieris brassicae* · Brassicaceae

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Introduction

Herbivory induces the emission of volatile organic compounds, which are called herbivore induced plant volatiles

(HIPVs). Such chemicals are involved in plant indirect defense when they are used by the herbivores' natural enemies to locate their host or prey (e.g., Dicke and Sabelis, 1987; Geervliet et al., 1994; Turlings et al., 1995; Dicke and van Loon, 2000; Heil, 2008). The HIPV blends resulting from herbivore damage are not identical to those elicited by mechanical damage or released by intact plants (reviewed by Turlings et al., 1995; Takabayashi and Dicke, 1996). Indeed, mechanical wounding and chemical elicitors from insect oral secretions synergistically activate plant HIPV emissions (reviewed by Arimura et al., 2009). HIPVs are complex mixtures commonly consisting of 20 to over 200 compounds, ranging from simple alkanes to more elaborate structures such as sesquiterpenes (Dudareva et al., 2004). Such complexity may provide specific information to carnivorous arthropods and result in reliable cues for detection of suitable host or prey. The composition of the blend may vary according to plant and herbivore species, conditions, and developmental stage (Takabayashi et al., 1995; De Moraes et al., 1998; Gouinguené et al., 2003). Most published papers have described the role of HIPVs in simple tritrophic interactions that involve one species on each trophic level in the aboveground compartment of a plant system. However, roots can also emit HIPVs that attract enemies of belowground feeding herbivores (Aratchige et al., 2004; Rasmann et al., 2005; Ali et al., 2010). Therefore, more complex networks involving multiple herbivores should be studied (see Bezemer and van Dam, 2005; Dicke et al., 2009 for reviews). Feeding damage caused by root and shoot herbivores may lead to quantitative and qualitative changes of primary and secondary metabolites in both compartments of the plant (Bezemer and van Dam, 2005; van Dam et al., 2009). Such changes not only affect the development of herbivores in the two spatially separated compartments, but also the organisms on the third trophic level associated with these plants (Gols and Harvey, 2009). For instance, simultaneous feeding by aboveground herbivores (*Spodoptera littoralis*) and below-ground herbivores (*Diabrotica virgifera virgifera*) in maize plants affects the production of a single HIPV, (*E*)-β-caryophyllene, which in turn impacts on the behavior of respective natural enemies. This altered emission of volatiles reduced the attraction of the parasitic nematode that attacks the belowground herbivore (Rasmann and Turlings, 2007). Such changes in individual HIPV compounds may function synergistically with other less specific components as reliable cues for carnivores. For example, the homoterpene dimethyl-tridecatetraene (DMTT), is emitted by prey-infested lima bean plants (*Phaseolus lunatus*) but is not attractive to the predatory mite *Phytoseilus persimilis* as a pure compound. However, when it is added to a blend of compounds emitted from a plant infested by non-prey herbivores, predatory mites are attracted (Dicke et al., 1990). Thus, the specificity of each induced odor blend may be a function of the mixture, quantity or the relative amounts of the volatile compounds (De Boer et al., 2004; Mumm and Hilker, 2005). Plant volatile emissions should always be considered in the context of all the other volatiles of the blend (van Dam and Poppy, 2008; van Dam et al., 2010; Ranganathan and Borges, 2010). HIPV emissions thus require multivariate analysis to allow understanding in terms of insect behavior. Furthermore, herbivores not only increase the emission of certain compounds but also can reduce the release of some wound-elicited constituents (Gaquerel et al., 2009). Although there are few examples in the literature, blends of HIPVs also may differ qualitatively and then provide specificity of chemical cues in host location by parasitoids (Takabayashi et al., 2006). Both quantity and quality of the volatiles perceived by natural enemies may determine the reliability of the informative odor (Vet et al., 1991).

Delia radicum, the cabbage root fly, is a major pest of brassicaceous crops. Females aggregatively lay their eggs near plant stems, and the larvae crawl down to feed on the roots and develop until they pupate in the soil. These larvae frequently are parasitized by *Trybliographa rapae*, a specific larval endoparasitoid of *Delia* species. This parasitoid forages aboveground for hosts that are feeding on plant roots belowground; therefore, systemically emitted volatiles play a key role in its host location. Females are attracted by volatiles emitted by undamaged leaves of *D. radicum* infested plants but not by volatiles emitted by leaves of artificially damaged plants (Neveu et al., 2002). Furthermore, volatiles emitted by leaves of plants artificially damaged at the roots to which crushed larvae are applied are attractive; this suggests that the attractive blend of HIPVs produced is specific to *D. radicum* feeding activity (Neveu et al., 2002). The specific composition of the HIPV blend elicited by *D. radicum* feeding on turnips and involved in *T. rapae* attraction has not been determined. A recent study showed that when *Pieris brassicae* (a specialist leaf feeder of brassicaceous plants) and *D. radicum* simultaneously attack a turnip plant (*Brassica rapa* subsp. *rapa*), *T. rapae* is no longer attracted (Pierre et al., 2011). Reduced attraction of root and shoot infested plants in olfactometer experiments was correlated with lower levels of parasitism in a separate field study (Pierre et al., 2011).

In this study, we analyzed the blends of HIPVs emitted by turnip plants after (1) infestation by *D. radicum*, (2) infestation by *P. brassicae*, (3) simultaneous infestation by *D. radicum* and *P. brassicae*, or (4) no infestation. The combination of experiments revealed specific modifications of HIPV blends following single and dual infestations. This is a first step towards understanding the altered foraging behavior of *T. rapae* in the presence of leaf herbivory. The analysis of HIPVs needs a multivariate approach in which

the relative amounts of volatiles are as important as the amounts themselves. Therefore, Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) was used to observe the effect of different forms of herbivore infestation on HIPV emission blends. The resulting model gives the relative importance of each volatile in a system-wide model describing the entire blend. It optimally describes the effect of each infestation as a classification between control and treated plants. However, OPLS-DA does not assess differences in the patterns of HIPV emissions between single herbivore infestations and dual herbivory. So it does not explicitly reveal the synergistic or antagonistic effects of additional herbivory. To address this question, univariate analysis was used to evaluate *a posteriori* the interaction between leaf and root herbivory effects on the levels of important HIPVs.

Method and Materials

Plants Cultivated turnip plants, *Brassica rapa* subsp. *rapa* (var. Nancy) were used. Seeds were sown in compost-soil, and after 10 days, seedlings were transplanted into 9×9× 9 cm plastic pots, filled with sandy soil. Plants were grown in a greenhouse, at 60% r.h., 21°C/16°C, 16/8 h L:D photoperiod. Natural day-light was supplemented when needed with metal halide lamps (200 μmol/s/m^2 PAR = photosynthetically active radiation; 1 lamp per m^2). Plants were watered every day and supplemented with nutrients (4 N: 6P: 8 K and micronutrients) twice a week until they were 7- to 8–wk-old.

Insects Delia radicum larvae used for plant infestation originated from our rearing. The populations were originally established in the laboratory from flies collected in the field at St Méloir des Ondes (Brittany, France) during the summer of 1994. The brood was reared as described in Neveu et al. (1996) and supplemented yearly with new field-captured individuals coming from the same region. *Pieris brassicae* caterpillars originated from an insect culture maintained at the Laboratory of Entomology of Wageningen University, the Netherlands, and were reared on *Brassica oleracea* subsp. *gemmifera* (Brussel sprout) plants.

Induction Procedure Plants were infested when they had five to eight fully expanded leaves (7-8-wk old). Twelve to thirteen plants of similar size and stature were selected for each of the different treatment groups. Infestation with *P. brassicae* (P) consisted of placing ten first instars with a small brush on the two youngest leaves (5 per leaf) of each plant. Plants were infested by *D. radicum* (D) by placing 5 s instars with a brush onto the soil surface, immediately adjacent to the stem of each plant. Plants were checked 1 h later to control whether all larvae had disappeared into the sand. With dual infestation (DP), larvae of *P. brassicae* (see above) were applied first to each plant followed 1 h later by *D. radicum* larvae. A nutrient solution (4 N: 6P: 8 K and micronutrients) then was applied to both infested and uninfested plants.

Collection of Volatiles and GC-MS Analysis Volatiles were collected 3 days after induction by infestation. Based on our previous studies with turnip plants, 3 days of infestation sufficed for effective induction of *T. rapae* attracting volatiles (Neveu et al., 2002; Pierre et al., 2011). The entire plants (including pots and sand) were transferred to four separate 17 l glass bell-shaped collection chambers placed in a controlled climate cabinet (21°C, 70% r.h.). The chambers were supplied constantly at the top with pressurized air (Hoek Loos, NL) cleaned over a Zero Air generator to remove hydrocarbons (Parker Hannifin Corp, Tewksbury, MA, USA). Plant-generated HIPVs were collected over a steel trap filled with 150 mg Tenax TA and 150 mg Carbopack B by using a vacuum pump. Collection flow rates were set to 100 ml/min. The HIPVs were collected from an outlet on the side of the flask, which was at the same height as the plant leaves. The supply rate of clean air at the top of the flask was set to 200 ml/min. After 1 h, the traps were removed from the pump and capped until analysis. In this way, within 1 days we measured six full series of four plants representing the four different treatments tested and two background HIPV profiles from an empty glass chamber. We sampled the following numbers of individual plants: 13D, 13P, 12DP, and 13C. Volatiles were desorbed from the traps, then analyzed by GC-MS using the same methods and reference compounds as described by van Dam et al. (2010). All integrated signals were generated from the MS-chromatograms by the AMDIS software, except those of alpha-pinene, isobutyl isothiocyanate, and 2-isothiacyanato-butane, which were extracted as ion traces from the same peak from which they co-eluted by Xcalibur software. Note that these three compounds co-eluted so close that they could not be separated even by deconvolution. However, strong unique ions were found in the mass spectrum of the mixture (alpha-pinene, 93; isobutyl isothiocyanate, 115, 2-isothiacyanato-butane, 86) and relative trends generated from these single qualifier ions are in theory proportional to trends in the total ion traces.

Peak areas obtained in each sample were divided by the total volume in ml that was sampled in the trap, to correct for minor differences in sampling time and flow rates over individual traps.

Data Analysis of Volatile Profiles Peaks relating to mixtures and impurities were removed from the dataset.

Furthermore, to avoid rare compounds that disproportionably could affect the outcome, only those compounds that were found at least six times in at least one treatment group were retained for the multivariate analyses. This resulted in a dataset of 37 HIPVs for detailed analysis. We constructed three separate models, each of which compared the HIPV profiles of one form of herbivore infestation to the control plants, thereby isolating the effect of one herbivory form on the HIPV composition. For these models, the Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) method was chosen. It takes existing biochemical relations among the volatile compounds into account, and the interpretation of results from the model compare well in terms of the differences among the plant groups (Bylesjö et al., 2006). In brief, OPLS-DA is a supervised component analysis method that uses *a priori* knowledge about the form of herbivory that every plant received. The models consist of a small number of 'Latent Variables'—comparable to the components of Principal Component Analysis—which together describe the difference between control and treated plants. The models in this study consisted of a description of the treatment each plant most likely received (the class membership description, γ_{hat}) and the metabolic profile with relative importance of each volatile in the total blend (the OPLS-DA weight vector). Although OPLS-DA models may contain multiple latent variables, there is always only one class prediction and one weight vector *per* plant when two groups are compared.

The OPLS-DA model thereby specifically focuses on differences among plant groups, and like all supervised models it is thereby prone to 'over fit' these differences, i.e., describing the variation among the individual plants used in the experiment, rather than generic phenomena caused by the experimental manipulations (Jansen et al., 2010). Therefore, the observations from the resulting models were evaluated thoroughly by cross-validation, in which the data of randomly chosen individual plants are left out of the data set and the model is re-made, after which the treatment of the left-out plants is estimated. This procedure was repeated 2,000 times, which then leads to a misclassification rate; a measure of model error. The number of latent variables for which the misclassification rate is lowest results in a model that includes all systematic information useful for predicting treatment groups, but does not contain superfluous 'random' information that relates to over fit. As a second validation, the determined misclassification rate then was compared to that of a large number of models in which treatments were artificially shuffled by permutation: this naturally should lead to considerably higher misclassification, because true biological difference between the permuted treatments is lacking. The permutation is repeated 1,000 times, each time consisting of 2,000 cross-validated models providing a misclassification rate. The difference in misclassification rate between the model with real and permuted treatments can be expressed as a *P*-value that describes the likelihood that the observed effect is real.

The chemistry in the 'weight' vector of treatments with a significant effect on HIPV emissions was analyzed further. This vector—showing the importance of each volatile in describing the differences between two groups— was compared to those obtained from the models on permutated treatments described before. These permutated models collectively form a 'null distribution' of the OPLS-DA weights for every volatile, from which the weight of the model fitted on real treatments should differ. This last permutation analyses results in a *P*-value for every volatile for each form of herbivory.

In our case, there were three separate OPLS-DA models that we compared for communalities and differences caused by different forms of herbivory. This comparison was done by plotting the weight vectors of each volatile on the first latent variable (LV) for each treatment. However, three-dimensional figures that compare the different forms of herbivory are difficult to interpret. By condensing the relevant information to a two-dimensional figure, it becomes more insightful. This figure, called a bubble plot (Markus and Gu, 2010), compares the chemistry underlying two forms of herbivory as positions of each volatile on two axes (i.e., a scatter plot). In addition, the amount of volatile from the third form of herbivory is represented as the area of a circle: this graphical tool is useful for comparing both forms of individual herbivory and the third, combined infestation.

Afterwards, compounds of which the weight vector values were significant for at least one form of herbivory were selected for univariate analysis. Indeed, for each important compound, we tested the effect of root herbivory (D), leaf herbivory (P), and the interaction (D*P) by using two-way analysis of variance on peak areas transformed as log (x + 1) (van Dam et al., 2010). The significance of such an interaction is of interest, as it could reveal a positive or negative influence of dual herbivore treatment (DP) compared to a single herbivore one (P or D). A similar procedure was used previously by Soler et al. (2007a) to support the results of their multivariate analysis. In the present study, homogeneity of variance was checked by Levene's test using the "robvar" function in Stata 9 software (StataCorp LP, College Station, TX, USA).

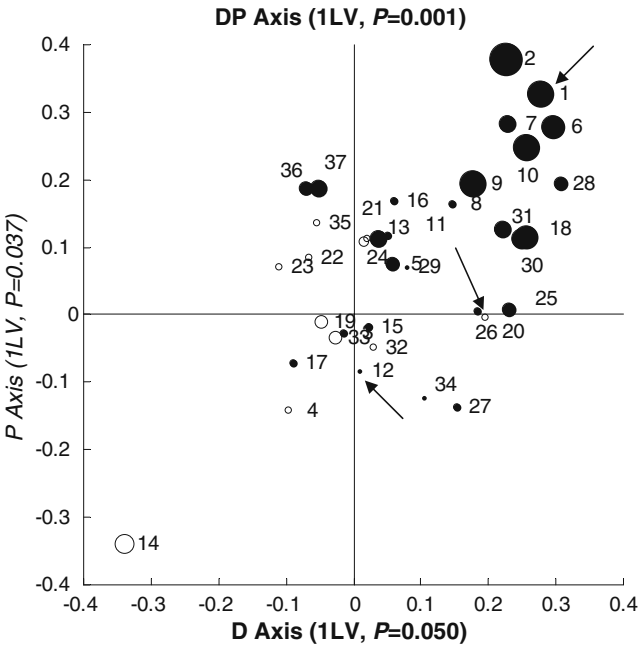
Results

The fitted OPLS-DA models showed that the effect of all three herbivore treatments on the HIPV emissions can be described

by simple low-dimensional multivariate models. The cross-validation yielded a single latent variable for all three to significantly distinguish the volatile blends emitted by turnip plants (Table in Fig. 1). The comparison of these three separate OPLS-DA models for communalities and differences caused by different forms and levels of herbivory was done by plotting the discriminate functions of root herbivory by *D. radicum* (D) and leaf herbivory by *P. brassicae* (P) against each other (Fig. 1). Increased levels of a compound in D or P are indicated by a positive weight vector value on the respective axis, while reduced levels are shown by a negative value. The size of each bubble corresponds to the weight vector value of a volatile for the dual herbivory treatment (DP). The maximal bubble size corresponds to (*3E*)-4,8-dimethyl-1,3,7-nonatriene DMNT (2), which then is related to the highest vector value for DP (0.394) and to the most significant difference from the vector value for the control (P=0.004). The absolute minimal bubble size is associated with heptadecene (29), which has the lowest absolute vector value for DP (0.002) and the least significant difference from the vector value for the control (P=0.595). The bubble size of hexylacetate (9) represents a threshold of significance (weight vector value=0.300; P=0.044) of weight vector values associated with DP.

For instance, the level of (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene TMTT (1) increased after any form of herbivory, as indicated by its high and positive weight vector value on the D axis (0.276), the P axis (0.329) and the large black bubble indicating a high and positive weight vector value for (DP) (0.305). By contrast, the level of 2-undecanone (20) was mildly increased by (D), which is represented by a relatively high and positive value in the D axis (0.196), but this did not respond for (P) and (DP) as indicated by a low weight vector in P axis (−0.004) and a small size of the bubble meaning a low weight vector value for (DP) (−0.055). An example of a compound whose level mildly decreased in (P) is 1-hexadecanol (12) which has a negative value on the P axis (−0.083); this compound did not respond to (D) or (DP), as revealed by the low value in the D axis (0.008) and the small size of the bubble (0.005) (Fig. 1).

Hence, the bubble plot representation allows visual identification of interesting compounds that are important for differentiating between D, P, and DP volatile profiles. In particular, the right upper quarter of the bubble plot shows the compounds, TMTT (1), DMNT (2), (*E,E*)-alpha-farnesene (6), hexylacetate (9), and (*E*)-3-hexen-ol-acetate (10) the levels of which were relatively high in all three herbivore treatments as indicated by the large filled dots (Fig. 1). Interestingly, in the same quarter of the figure, smaller black dots indicate compounds that increased in P or D but not in DP, i.e., methysalicylate (7) and 4-methyltridecane (28) (Fig. 1). Consequently, the latter



treatment	number of LVs	*P*-values
(D)	1	0.050
(P)	1	0.037
(DP)	1	0.001

Fig. 1 Two dimensional bubble plot representation of volatiles emitted after different forms of herbivore infestations in *Brassica rapa* subsp. *rapa* plants: discriminate functions of root herbivory by *Delia radicum larvae* (D) and leaf herbivory by *Pieris brassicae* caterpillars (P) plotted against each other. Numbers correspond to compounds listed in Table 1. The size of each bubble representing a volatile corresponds to the weight vector value of that volatile for the dual herbivory treatment, i.e., herbivory by *Delia radicum* larvae and *Pieris brassicae* caterpillars (DP). The *color* shows if the volatile increases (*filled*) or decreases (*open*) in the DP treatment. The *three arrows* indicate examples of compounds given in the text. The table below presents the number of latent variables (LVs) prescribed by cross-validation and the *P*-values for the significance ($P \leq 0.050$) of the system-wide effect brought about by each form of herbivory

compounds are compounds that would differentiate between single herbivory and dual herbivory effects on the plant volatile profile.

Volatile Profiles of Plants Exposed to Root Herbivory (D)

Permutation tests of weight vector values showed that the level of 4-methyltridecane (28) was higher in plants exposed to herbivory by D alone compared to control plants (Table 1). This compound appeared to be specific for the volatile blend emitted by (D), as its level was significantly modified in D plants but neither in P nor DP plants. The levels of the homoterpene TMTT (1) and the sesquiterpene (*E,E*)-alpha-farnesene (6) also were higher,

whereas that of the alcohol 2-octen-1-ol (14) was lower in plants exposed to (D) (Table 1). In addition to these significant differences, a marginal increase (arbitrary threshold for marginal variations: $P \leq 0.065$) was observed for salicylaldehyde (18) and the green leaf volatile (*E*)-3-hexen-1-ol-acetate (10) (Table 1). All those compounds appeared to be important in characterizing the volatile blend emitted by root infested plants, since their corresponding weight vectors had either high or low values compared to control plants (Fig. 1).

Volatile Profile of Plants Exposed to Leaf Herbivory (P) The level of methylsalycilate (7) significantly increased after (P) compared to control plants (Table 1). This phenomenon is specific for the odor blend emitted by leaf infested plants, as it does not appear in the other forms of herbivore treatment. Similar to (D), the volatile emissions of TMTT (1) and (*E,E*)-alpha-farnesene (6) significantly increased, whereas they decreased for 2-octen-1-ol (14) in plants exposed to (P) (Table 1). In addition, the levels of DMNT (2), and of the green leaf volatile (*E*)-3-hexen-1-ol acetate (ten marginally, i.e., $P \leq 0.065$) increased after (P) compared to control plants (Table 1).

Volatile Profile of Plants Exposed to Both Forms of Herbivory (DP) The level of hexylacetate (9) was significantly higher in (DP) compared to control plants (Table 1). This is specific for the volatile blend from dual infested plants. Indeed, no significant increase in hexylacetate was observed in (P) or (D), (Table 1). Furthermore, some compound levels in the headspace of DP followed patterns similar to single infested plants, either with D or P. This was true for increased levels of TMTT (1), (*E*)-3-hexen-1-ol-acetate (10) and, marginally so (i.e., $P \leq 0.065$) for (*E,E*)-alpha-farnesene (6) (Table 1). DMNT (2) also increased in DP plants, which followed the same tendency as P (Table 1). By contrast, lower levels of 2-octen-1-ol (14) were observed both in (D) and (P), but not in (DP), despite a similar tendency in the single infestations (Table 1).

In summary, both the bubble plot and permutation tests show that the plant response to dual herbivory (DP) is different from the combined effects of *D. radicum* and *P. brassicae* feeding alone (Fig. 1), despite a few communal patterns.

Effect of Interactions Between Root (D) and Leaf Herbivory (P) Only compounds of which the weight vector values were found to be significant for at least one form of herbivory were selected for univariate analysis to assess the interaction between (D) and (P) effect (Table 2). ANOVA analysis (Table 2) showed a significant interaction between (D) and (P) effects on 4-methyltridecane (28). This implies that the positive effect of (D) on the emission of this compound was reduced in the presence of (P) (Table 1, Table 2). Together with the bubble plot (mentioned above), such interaction indicates that the effect of root herbivory on this compound was different from the effect of dual herbivory. This confirms the high specificity of this compound for turnip plants infested by the root herbivore alone. In addition, an antagonistic effect between D and P was observed for 2-octen-1-ol (14; Table 1, Table 2). Indeed, the concentration of this compound was reduced in plants infested by D or P alone (as already mentioned) but not in dual infested plants.

The positive effect of P on the levels of methylsalycilate (7; Table 1) was significantly reduced by the additional presence of D. In other words, the effect of P was different from the effect of DP, as shown by the bubble plot (Fig. 1 mentioned above) and the significant interaction between D and P (Table 2). This confirms the specificity of methylsalycilate (7) induction as a cue for the leaf herbivores on plants without root damage. In addition, a significant interaction between D and P was observed for (*E,E*)-alpha-farnesene (6) (Tables 1, 2). The interaction was suggested by a marginal (i.e., $P \leq 0.065$) although not significantly higher level of (*E,E*)-alpha-farnesene in dual infested plants compared to control plants. Permutation tests revealed higher levels of hexylacetate (9) in DP compared to control plants (Table 1), which is specific for such herbivore treatment since no significant increases were observed in D and P plants. However, ANOVA did not show any significant effects of interaction between D and P on that compound.

Discussion

Our results show that higher levels of the branched alkane, 4-methyltridecane were highly specific for the volatile blend of plants infested by the root herbivore alone. Different alkanes, including branched alkanes, already have been detected in leaf and floral headspace of brassicaceous plants (Jakobsen et al., 1994; Gruber et al., 2009). However, we are unaware of any publications on the specific involvement of alkanes in the attraction of natural enemies of herbivores. In addition, salicylaldehyde emissions were marginally ($P=0.058$) modified by root herbivore feeding. Salicylaldehyde already has been reported to play an important role in some tritrophic systems that involve leaf beetle larvae (*Chrysomela* spp.) and their natural enemies (reviewed by Gross and Schmidtberg, 2009). The two specific HIPVs, 4-methyltridecane and salicylaldedhyde, thus, could serve as cues for the parasitoid *T. rapae* when searching for *D. radicum* infested plants without leaf herbivores present as well. Further investigations based for example on gas chromatography coupled to electro-antennography (GC-EAG) could reveal which compounds

Table 1 Peak areas transformed as log (X+1) of volatile compounds from *Brassica rapa* subsp. *rapa* plants exposed to root herbivory by *Delia radicum* (D) or leaf herbivory by *Pieris brassicae* (P), dual herbivory by *Delia radicum* and *Pieris brassicae* (DP), or no herbivory (control)

No.	Compound name[a]	RI[b]	Compound class	Control	D	P	DP	*P* (D)[c]	*P* (P)	*P* (DP)
1	**(*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT)**	1577	Homoterpene	0.00±0.00	0.87±0.38	1.52±0.41	1.17±0.42	**0.046**	**0.011**	**0.022**
2	**(3*E*)-4,8-Dimethyl-1,3,7-nonatriene (DMNT)**	1117	Homoterpene	0.92±0.40	2.06±0.48	3.32±0.32	3.10±0.30	0.108	**0.004**	**0.004**
3	alpha-Pinene*	932	Monoterpene	1.89±0.08	1.88±0.04	1.86±0.07	1.91±0.06	0.483	0.448	0.427
4	Limonene*	1026	Monoterpene	3.33±0.09	3.25±0.05	2.97±0.26	3.26±0.07	0.311	0.268	0.319
5	Caryophyllene	1413	Sesquiterpene	0.83±0.44	1.11±0.48	1.27±0.46	1.85±0.57	0.443	0.339	0.151
6	**(*E,E*)-alpha-Farnesene***	1506	Sesquiterpene	2.11±0.41	3.43±0.33	3.69±0.35	3.39±0.36	**0.023**	**0.044**	0.055
7	**Methylsalicylate**	1191	Ester	1.00±0.37	1.97±0.39	2.45±0.32	1.87±0.41	0.111	**0.046**	0.145
8	Ethylacetate	612	Ester	1.94±0.61	2.87±0.55	3.16±0.50	2.09±0.63	0.226	0.126	0.492
9	**Hexylactetate**	1015	Ester	2.29±0.37	2.92±0.27	3.17±0.29	3.42±0.10	0.178	0.113	**0.044**
10	**(*Z*)-3-Hexen-1-ol-acetate**	1008	Ester	4.36±0.13	4.71±0.10	4.82±0.13	4.88±0.11	0.054	0.061	**0.016**
11	1-Tetradecanol	1675	Alcohol	1.47±0.46	1.72±0.46	2.13±0.42	1.76±0.45	0.425	0.237	0.417
12	1-Hexadecanol	1881	Alcohol	2.91±0.26	2.94±0.38	2.53±0.43	2.93±0.28	0.465	0.301	0.488
13	3-Hexen-1-ol	850	Alcohol	2.99±0.37	3.07±0.39	3.49±0.30	2.54±0.54	0.404	0.237	0.251
14	**2-Octen-1-ol**	1068	Alcohol	2.08±0.34	0.64±0.34	0.40±0.27	0.92±0.40	**0.014**	**0.008**	0.075
15	1-Hexanol	865	Alcohol	2.50±0.21	2.55±0.22	2.44±0.31	2.63±0.24	0.359	0.500	0.311
16	1-Octanol	1071	Alcohol	2.71±0.34	2.90±0.25	3.25±0.05	2.94±0.27	0.468	0.230	0.453
17	1-Dodecanol	1474	Alcohol	3.06±0.14	2.84±0.28	2.86±0.27	3.08±0.11	0.311	0.371	0.473
18	Salicylaldehyde	1038	Aldehyde	0.49±0.26	1.33±0.31	0.91±0.30	1.38±0.30	0.058	0.233	0.080
19	Octadecanal	2021	Aldehyde	1.95±0.45	1.72±0.47	1.89±0.44	1.03±0.44	0.455	0.486	0.161
20	2-Undecanone	1293	Ketone	0.91±0.40	1.80±0.41	0.89±0.39	0.67±0.35	0.122	0.483	0.376
21	2,4-Pentadione	789	Ketone	2.47±0.48	2.67±0.52	3.12±0.40	3.33±0.31	0.386	0.280	0.185
22	(*E*)-Geranyl acetone	1451	Ketone	3.74±0.05	3.71±0.06	3.81±0.07	3.70±0.06	0.374	0.318	0.323
23	2-Nonanone	1091	Ketone	2.28±0.29	1.88±0.37	2.51±0.22	2.17±0.30	0.303	0.379	0.406
24	Acetone	516	Ketone	3.78±0.60	3.88±0.61	4.48±0.38	2.88±0.74	0.400	0.309	0.269
25	6-Methyldodecane	1253a	Alkane	1.60±0.43	2.61±0.33	1.64±0.44	2.32±0.41	0.092	0.424	0.204
26	3-Methylundecane	1176a	Alkane	1.77±0.41	2.51±0.31	1.8±0.41	1.95±0.42	0.167	0.455	0.421
27	2,6-Dimethylundecane	1212a	Alkane	1.56±0.49	2.36±0.46	0.76±0.40	1.99±0.51	0.209	0.215	0.382
28	**4-Methyltridecane**	1358a	Alkane	1.47±0.40	2.68±0.23	2.41±0.30	2.18±0.38	**0.026**	0.089	0.194
29	Heptadecene	1692	Alkene	1.33±0.49	1.74±0.47	1.77±0.48	1.34±0.48	0.312	0.338	0.595
30	2,4-Dithiapentane	882	Sulfide	0.48±0.33	1.58±0.43	1.06±0.46	1.53±0.48	0.074	0.301	0.111
31	Dimethyldisulfide*	738	Sulfide	3.08±0.28	3.62±0.11	3.43±0.11	3.60±0.09	0.101	0.289	0.138
32	Hexanoic acid	981	Acid	1.50±0.41	1.63±0.44	1.26±0.39	1.13±0.41	0.414	0.415	0.334
33	Acetic acid	568	Acid	3.50±0.44	3.36±0.53	3.30±0.53	2.72±0.59	0.473	0.48	0.245
34	Indole*	1287	Indole	1.31±0.35	1.69±0.33	0.77±0.33	1.34±0.35	0.299	0.203	0.508
35	Dihydroxybenzoquinone (DBQ)	1462	Unknown	1.80±0.42	1.56±0.42	2.47±0.32	1.49±0.45	0.408	0.193	0.359
36	Isobutyl isothiocyanate	931	Isothiocyanate	1.06±0.15	0.94±0.16	1.47±0.17	1.27±0.10	0.341	0.114	0.201
37	2-Isothiocyanato-butane	920	Isothiocyanate	0.66±0.15	0.58±0.15	1.09±0.19	0.91±0.09	0.376	0.129	0.142

[a] The table contains those volatile organic compounds (VOCs) that were retained for multivariate analysis. Compounds indicated with a * were identified by comparison of pure standards, the other compounds were tentatively identified according to the same references as cited by van Dam et al. (2010). The numbers in the first column refer to the numbers in the two dimensional bubble plot in Fig. 1

[b] RI = retention index on the GC-MS; column RTX5-MS. Peak areas of compounds are means±SE

[c] The *P*-values were determined from a permutation test of the weight vector value which reflects the importance of each compound in the difference between herbivory by one form of herbivory and the control: root herbivory by *Delia radicum* larvae = *P* (D); leaf herbivory by *Pieris brassicae* caterpillars = *P* (P); dual herbivory by *Delia radicum* larvae and *Pieris brassicae* caterpillars = *P* (DP). Bold face type indicate compounds for which the weight vector value is significant for a specific treatment ($P \leq 0.050$)

Table 2 *P*-values (*P*(D*P)) from two way analysis of variance on peak areas transformed as log (X+1), testing the effect of the interaction between root herbivory by *Delia radicum* larvae (D) and leaf herbivory by *Pieris brassicae* caterpillars (P) treatments of *Brassica rapa* subsp. *rapa* plants for each selected volatile organic compound

No.	Compound name	*P* (D*P)
1	(*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	0.084
2	(3*E*)-4,8-Dimethyl-1,3,7-nonatriene	0.083
6	(*E,E*)-alpha-Farnesene	**0.032**
7	Methylsalicylate	**0.041**
9	Hexylacetate	0.490
10	(*Z*)-3-Hexen-1-ol-acetate	0.223
14	2-Octen-1-ol	**0.005**
28	4-Methyltridecane	**0.037**

The numbers in the first column refer to the numbers in the two dimensional bubble plot in Fig. 1. The table contains those compounds of which the weight vector values refered in Table 1 were significant for at least one form of herbivory. Bold face types indicated significant *P*-values ($P \leq 0.050$)

within the blend of HIPVs elicit a response in the chemoreceptors of the parasitoid (Dicke and Baldwin, 2010).

The volatile blend of plants after leaf herbivory by *P. brassicae* was characterized by higher levels of methylsalicylate. The herbivore-induced emissions of this compound were annulled in the presence of the root herbivore. This volatile is known to be active at very low levels (Gouinguené et al., 2005), attractive for carnivorous arthropods under laboratory conditions (Dicke et al., 1990; Ozawa et al., 2000; De Boer and Dicke, 2004), and for hymenopteran parasitoids in the field (James and Price, 2004; Poelman et al., 2009). On the other hand, methylsalicylate diminished the attraction of the parasitoid *Diadegma semiclausum*, a specialist of another *Brassica* specialist herbivore (*Plutella xylostella*) in a mutant of *Arabidopsis* (Snoeren et al., 2010). Therefore, the lack of attraction of *T. rapae* towards *P. brassicae* infested plants compared to root infested plants (Pierre et al., 2011) could be attributed at least partly to a higher level of methylsalicylate and/or to lower levels of 4-methyltridecane and salicylaldehyde.

Dual infested plants exhibited a specific increase of one compound, the green leaf volatile hexylacetate. Specific compounds that were induced by leaf or root herbivory (i.e., 4-methyltridecane and methylsalycilate) did not show changes in emission levels after dual herbivory. Therefore, it appears that simultaneous herbivory significantly reduces or even suppresses the induction of specific compounds that characterize the volatile profile of single infested plants. Only one compound, 2-octen-1-ol, decreased after both single herbivory treatments, but not after dual herbivory.

In addition to specific variations, we also observed communal trends in the volatile profiles among the three forms of herbivory. In particular, the levels of the terpenoids TMTT and (*E,E*)-alpha-farnesene (marginally induced in dual infested plants) increased after any form of herbivory. Furthermore, the levels of the terpenoid DMNT increased after infestation by the leaf herbivore only, or after dual infestation. These terpenoid compounds are attractive for herbivorous and carnivorous (especially parasitoid) insects associated with brassicaceous plants (e.g., Soler et al., 2007a; van Dam et al., 2010). The present study shows that the specificity of each volatile blend may be due to a mixture of a few specific compounds that increase within the more common odor context of terpenoids and green leaf volatiles. The potential role of such background odors in the use of volatiles by foraging parasitoids has already been reviewed (Hilker and McNeil, 2007). In brassicaceous plants, terpenoids and green leaf volatiles often are abundant within HIPV mixtures, irrespective of the herbivore species that damages the plant. Thus, they themselves may not provide specific cues that allow specialist parasitoids to identify their hosts, which suggests that the specificity of the odor cue then may lie in ratios of the emitted volatiles within the blend (Gols and Harvey, 2009). In this way, the minor quantitative changes in dual infested plants may have contributed to the lack of attractiveness towards *T. rapae* and to the lower levels of parasitism of *D. radicum* in the field (Pierre et al., 2011). The reduced attractiveness of *T. rapae* towards dual infested plants may be adaptive, as both survival and size of *T. rapae* developing in *D. radicum* on plants infested by *P. brassicae* are reduced (Soler et al., 2007b). The combination of the host *D. radicum* and the non-host *P. brassicae* on the same plant often occurs in the field (Pierre et al., 2011), and it is likely that the parasitoid has learned to detect the characteristic volatile blend of this combination. Similar trends also were found for reverse interactions: root herbivory by *D. radicum* altered the foraging and egg laying behavior of *C. glomerata*, a parasitoid of *P. brassicae* (Soler et al., 2007a). In this case, the volatile blend of dual infested plants also differed only by a few compounds; the emissions of two sulfur compounds were higher, whereas two terpenoids were less prominent in dual infested plants (Soler et al., 2007a). These minor modifications of the complex plant odor blend were hypothesized to be sufficient for the aboveground parasitoids to avoid plants with root herbivores.

The mechanisms involved in the non-additive modification of the odor blends emitted by *D. radicum* and *P. brassicae* infested turnips remain to be elucidated. Crosstalk between signaling hormones, such as jasmonic acid and salicylic acid, involved in responses to different herbivore species may have contributed (van Poecke and Dicke, 2002; Erb et al., 2008; Koornneef and Pieterse, 2008; Zhang et al., 2009). Alternatively, interference

between HIPV biosynthetic pathway activation by root and shoot herbivory may be involved. A recent study suggested that root induced responses are regulated fundamentally differently from responses triggered by shoot herbivory (van Dam et al., 2010). It was found that the biosynthesis of sesqui- and homoteperne volatiles was induced only by shoot jasmonic acid treatment of *B. oleraceae* plants, whereas monoterpenes were equally elicited by root and shoot jasmonic acid treatment. In the present study, we observed an interaction between root herbivory and leaf herbivory at the level of sesquiterpene (*E,E*)-alpha-farnesene. Therefore, these results support the hypothesis that root and shoot plant responses interfere, leading to variations in the dual infested plant odor mixture. A molecular approach that analyzes the expression of genes coding for markers of signaling or biosynthetic pathways could help to reveal which one is activated or suppressed in response to dual herbivory. Our chemical analysis supports previous behavioral studies in the multitrophic system, turnip-*D. radicum-P. brassicae-T. rapae.* Attraction of the parasitoid towards host infested plants was cancelled when these plants were also infested by the leaf feeder *P. brassicae* (Pierre et al., 2011). Analysis of volatile profiles revealed minor quantitative differences between plants infested by a single herbivore species and dual infested plants. However, these subtle changes were found to be highly specific, and thus may be sufficient for *T. rapae* to discriminate between the different HIPV mixtures. Our data interpretation using OPLS-DA, a novel multivariate analysis approach, has enabled us to identify the minimal, but relevant, changes within a complex HIPV background. This approach provides insight into the information processing of insects that base their behavioral responses upon quantitative differences in HIPV profiles as well as on qualitative contrasts within the context of an odor blend (Hilker and Mcneil, 2007; van Dam and Poppy, 2008). However, additional bioassays coupled with chemical analysis are required to conclude with certainty which compounds or combination of compounds within the plant volatile blend are behaviorally active towards the parasitoid *T. rapae*.

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Specificity of Induced Resistance in Tomato Against Specialist Lepidopteran and Coleopteran Species

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Abstract When challenged by herbivorous insects, plants produce a suite of antinutritive proteins that disrupt digestion and absorption of essential nutrients by the insects. We hypothesized that plants would induce distinct defense responses corresponding to the distinct midgut conditions of different herbivores. We investigated whether or not tomato responds specifically to two specialist herbivores: Colorado potato beetle (CPB; *Leptinotarsa decemlineata; C*oleoptera: Chrysomelidae) and tobacco hornworm (THW; *Manduca sexta*; Lepidoptera: Sphingidae), and we evaluated whether the induced defenses triggered by either species affect CPB growth. Tomato did not induce different defense genes in response to CPB or THW but accumulated more transcripts for some defense genes after damage by THW feeding compared to damage by CPB feeding. In addition, trypsin protease inhibitor activity and polyphenol oxidase activity were higher in plants damaged by THW than in plants damaged by CPB. Application of oral secretions from THW to wounded tomato plants increased transcripts compared to controls, but oral secretions from CPB decreased defense transcripts. CPB growth was compromised on plants damaged by either species, suggesting a low specificity of effect. Together, these data suggest distinct quantitative responses of tomato to two different specialist herbivores. Herbivore oral secretions might be responsible for these species-specific responses.

Key Words Oral secretions · Plant defense · Tomato · Colorado potato beetle · Tobacco hornworm · Protease inhibitors · Induced resistance · Specificity

Introduction

Plants have evolved a wide array of defense mechanisms against insect herbivores. Some of these include physical defenses (e.g., thorns) or chemical toxins (e.g., alkaloids) that are expressed constitutively, but others are induced in response to herbivore attack. Induced defenses can be categorized into two groups: (1) direct defenses, including induced toxins, trichomes, and antinutritive proteins, and (2) indirect defenses, including emission of volatiles that attract natural enemies (Howe and Jander, 2008). The specificity of induced responses may be an important trait to cope with a variety of stimuli.

Specificity of induced responses to herbivores comprises two parts: the specificity of elicitation and the specificity of effect (Karban and Baldwin, 1997; Stout et al., 1998). Specificity of elicitation is defined as distinct defense responses against different herbivores. For example, chewing and phloem-feeding insects cause different damage on plants and induce different suites of plant responses (Walling, 2000). In tomato, defense proteins induced by tomato fruitworm (*Helicoverpa zea*) feeding are different from those induced by potato aphid (*Macrosiphum euphorbiae*) feeding (Stout et al., 1998). Feeding by insects in the same feeding guild also may show different responses. The tobacco hornworm (THW; *Manduca sexta*) elicited different phytohormones and transcriptional responses compared to the beet armyworm (*Spodoptera exigua)* (Voelckel and Baldwin, 2004; Diezel et al., 2009). Specificity of effect occurs when induced defenses have different effects on the

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growth of an herbivore. For example, the growth of *S. exigua* was reduced by consumption of plants damaged by conspecific species compared to consumption of the control or aphid-treated plants (Rodriguez-Saona et al., 2005).

Insect feeding damages tissue and introduces herbivore-derived compounds that may elicit defensive responses. The specificity of plant responses to herbivores may be determined by insect elicitors or herbivore-associated molecular patterns and by the feeding pattern of herbivores (Felton and Tumlinson, 2008; Mithöfer and Boland, 2008). Elicitors trigger biosynthesis of wound hormones including jasmonic acid and ethylene, induction of defense genes, and release of volatile emissions. In addition to insect elicitors, feeding patterns of herbivores play a key role in the specificity of induced responses. In lima beans, continuous mechanical wounding alone is sufficient to mimic volatile emissions produced by caterpillar damage (Mithöfer et al., 2005).

The specificity of response and effect may be revealed through the expression of induced proteins. Antinutritive proteins such as proteinase inhibitors (PIs), arginase (ARG), threonine deaminase (TD), or polyphenol oxidase (PPO) can be induced by mechanical wounding, insect feeding, or jasmonic acid (Ryan, 2000; Felton, 2005; Chen et al., 2005, 2007; Zhu-Salzman et al., 2008). PIs inhibit insect digestive proteases and disrupt absorption of essential nutrients (Ryan, 1990). PIs are categorized into several groups such as serine protease inhibitor (SerPI), cysteine protease inhibitor (CysPI), and aspartic protease inhibitor (AspPI) according to the protease they inhibit. Induction of PIs could be herbivore-specific to produce defensive proteins that are effective against specific proteases or in specific gut environments characteristic of the herbivore. ARG and TD degrade essential amino acids and retard growth of herbivores with alkaline midguts (Chen et al., 2005, 2007). PPOs catalyze the oxidation of phenolics to quinones and diminish the nutritional value of ingested proteins especially in herbivores with alkaline midguts (Felton et al., 1992).

Coleopteran species often have an acidic midgut and use cysteine and aspartic proteases as the major proteases for protein digestion (Wolfson and Murdock, 1987; Felton et al., 1992). In contrast, lepidopteran larvae normally have an alkaline midgut and rely on serine proteases (Felton and Duffey, 1991). Therefore, CysPI and AspPI would be predicted to have little effect on lepidopteran species, and SerPI should be less effective on coleopteran species (Zhu-Salzman et al., 2008). We hypothesized that plants may produce distinct suites of antinutritive proteins in response to specific attacking herbivores, and that production of SerPI, ARG, TD, and PPOs would increase in plants damaged by a lepidopteran species compared to plants damaged by a coleopteran species.

In this investigation, we tested whether the induced responses of tomato *Solanum lycopersicum* are specific to two different herbivore species: THW (*M. sexta*) and the Colorado potato beetle (CPB; *Leptinotarsa decemlineata*). Both species are specialist herbivores on Solanaceous plants such as tomato. To test specificity of elicitation, we evaluated whether tomato plants damaged by CPB accumulate different transcripts and have different protein activities compared to plants damaged by THW feeding. To test specificity of effect, we evaluated whether larval CPB growth is compromised more on plants damaged by CPB than on plants damaged by THW.

Methods and Materials

Plants, Insects and Oral Secretions Tomato (*Solanum lycopersicum* cv Better Boy) plants grown in Pro-mix potting soil (Premier Horticulture, Quakertown, PA, USA) were watered every day and fertilized with 3 g of Osmocote plus (15-9-12, Scotts, Marysville, OH, USA) until they reached the four-leaf stage. All leaf samples were frozen immediately in liquid nitrogen and stored at −80°C until analysis. Eggs of THW were purchased from Carolina Biological Supply (Burlington, NC, USA) and hatched in a growth chamber at 27°C with 16:8 hL: D, and larvae were reared on artificial diet. Adult CPB were collected from potato fields in Hamburg, PA, USA (Dr. Jim Steffel, Lehigh Agricultural & Biological Services, Inc., Hamburg, PA, USA) and were maintained on tomato plants in a greenhouse with 16:8 hL: D. Eggs of CPB were hatched in a growth chamber, and larvae were supplied with detached tomato foliage until pupation. Oral secretions were collected from fourth-instar larvae of THW and CPB with a Pipetteman micropipette (Gilson, Middleton, WI, USA) by gently disturbing the larval mouth. Crude oral secretion was centrifuged at 10,000 x *g* for 10 min to remove particulates and stored at −80°C until used.

Herbivore Treatments To compare gene expression of plants damaged by CPB and THW, one first-instar of CPB or THW was confined to a clip cage (diam: 2 cm) and allowed to feed for 24 hr, followed by removal of the insect. The damaged leaflets were scanned to measure the area consumed using SigmaScan Pro 5 (SPSS) and approximately 100 mg of leaf tissue were then harvested from damaged leaves. Control plants included undamaged plants, and negative control plants had empty cages.

To investigate the effects of CPB and TWH feeding on trypsin protease inhibitor activity, one first instar of CPB or THW was confined in a clip cage on the third leaf for 24 hr, resulting in consumption of 25 to 50% of available leaf area, based on visual estimates. The insect was removed,

and 48 hr later leaves were collected (72 hr after placing insects on plants). Empty cages were used for the negative controls, and no cages were used on the control plants.

To determine the effects of larval oral secretions on gene expression, plants were wounded using pipette tips to punch two holes next to the midveins of leaves, removing 0.26 cm^2 of tissue from each leaf. Oral secretions were diluted with 4 volumes of water before applying 20 µl to wounded areas on the experimental plants, or water on the wounded control plants. After 12 hr, leaf samples were harvested.

RNA Extraction and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) RNA was extracted and purified with an RNeasy Plus Mini-kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized using 1 µg RNA as the template (High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Foster City, CA). The Power SYBR Green PCR Master Mix and 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) were used to carry out qRT-PCR under following conditions: 95°C for 10 min, then 40 cycles of 15 sec at 95°C, 60 sec at 60°C. Gene-specific primers for *SerPI, CysPI, AspPI, ARG2, TD2,* and *UBQ* were used (Table 1) with dissociation curve analysis to confirm specificity of each primer. The expression levels of each genes were normalized relative to the housekeeping gene, ubiquitin, and were calculated using $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen, 2001). Efficiencies of the amplification reaction for genes tested and validation data for reference gene are described in Supplemental Tables 1, 2 and Supplemental Fig. 1.

Trypsin Protease Inhibitor (PI) Activity The activity of trypsin PI was measured in leaves using an assay for trypsin activity (Worthington, 1988). A 50 mg sample of leaf tissue treated as described above was ground in liquid nitrogen and extracted with 1.25 ml buffer (0.046 M Tris buffer, pH 8.1, 0.0115 M $CaCl_2$) containing 5% (w/v) insoluble polyvinyl-polypyrrolidone. Samples were centrifuged at 11,000 x *g* for 10 min at 4°C, and 20 µl of the supernatant were mixed with 10 µl of 1 mM HCl containing 0.4 µg bovine trypsin (Sigma, St Louis, MO, USA; T1426) and 70 µl of extraction buffer. The mixture was incubated for 10 min at room temperature, mixed with 100 µl of 2 mM p-toluene-sulfonyl-L-arginine methyl ester (Sigma, T4626), and monitored for 5 min at 247 nm. Trypsin PI activity was calculated as PI (%)=[1-A/C] x 100, where A represents trypsin activity of the sample and C represents the maximum value of tryspin activity in control plants without clip cages.

Feeding Assay To investigate whether defenses induced by prior feeding negatively affect insect performance, CPB larval growth was determined. Two first-instars of THW or five first-instars of CPB were confined in clip cages on the terminal leaflet of the 3 rd leaf and allowed to feed for 24 hr, resulting in consumption of 50 to 100% of the leaf area in each cage. Empty cages were clipped on control plants. Four days after initiating insect feeding the damaged leaflets were excised, and one first-instar CPB was placed on each damaged leaflet in 1 oz diet cup containing 1% agar. The fresh mass of each larva was measured 6 d later. PPO activity was measured on a 50 mg subsample of tissue from each treatment collected 4 d after the initial treatment.

Polyphenol Oxidase (PPO) Activity The tissue was ground in liquid nitrogen, extracted with 1.25 ml buffer (0.1 M potassium phosphate buffer, pH 7.0) containing 5% insoluble polyvinylpolypyrrolidone, and centrifuged at 11,000 x g for 10 min at 4°C. Five microliters of supernatant were mixed with 200 µl of 3 mM caffeic acid

Table 1 Primer lists for qRT-PCR

Gene name	Description	Genebank Accession No.	Forward/reverse sequence	Reference
SerPI	Serine proteinase inhibitor/ Proteinase inhibitor 2	K03291	GGATTTAGCGGACTTCCT TCT G/ ATGCCAAGGCTTGTACTAGAGAATG	(Graham et al., 1985)
CysPI	Cysteine proteinase inhibitor/ Cystatin	AF198390	GGTGAAGGAATGGGAGGACTTCAA/ GGAGGTTTGGGAATGGAACATTGG	(Girard et al., 2007)
AspPI	Aspartic proteinase inhibitor	SGN-U143342[a]	AGCCAGTCCTTGACACAAGTGGTA/ AGGTACACATCACCACCTAACGCA	(Uppalapati et al., 2005)
ARG2	Arginase 2	AY656838	TGGTGAAGGTGTAAAGGGCGTGTA/ TTACCAGCTTCGCAGCAACCATTG	(Chen et al., 2004)
TD2	Threonine Deaminase 2	M61915	CCCTGGGAGGTGATGTAGTTCT/ TCGAATGGTGGGATGTATTTGAG	(Samach et al., 1991)
UBQ	Ubiquitin	X58253	GCCAAGATCCAGGACAAGGA/ GCTGCTTTCCGGCGA AA	(Rotenberg et al., 2006)

[a] Solanaceous Gene Network (SGN) identifier

(Sigma, Cat. No. C0625), and absorbance at 450 nm was monitored for 5 min. PPO activity was expressed as change in absorbance/min/mg tissue (Felton et al., 1989).

Statistical Analyses Transcript levels of defense genes and trypsin PI activities were analyzed by the non-paramatric Kruskal-Wallis and Tukey multiple comparison. Larval weight and PPO activities were analyzed by one-way ANOVA followed by Tukey multiple comparison. Weight data and PPO activities were log-transformed to meet assumptions of normality and homogeneity of ANOVA. All analyses were conducted with SAS 9.2.

Results

Gene Expression and Trypsin PI Activity CPB and THW feeding for 24 hr in a clip cage increased expression levels of defense genes compared to the control treatment (Fig. 1;

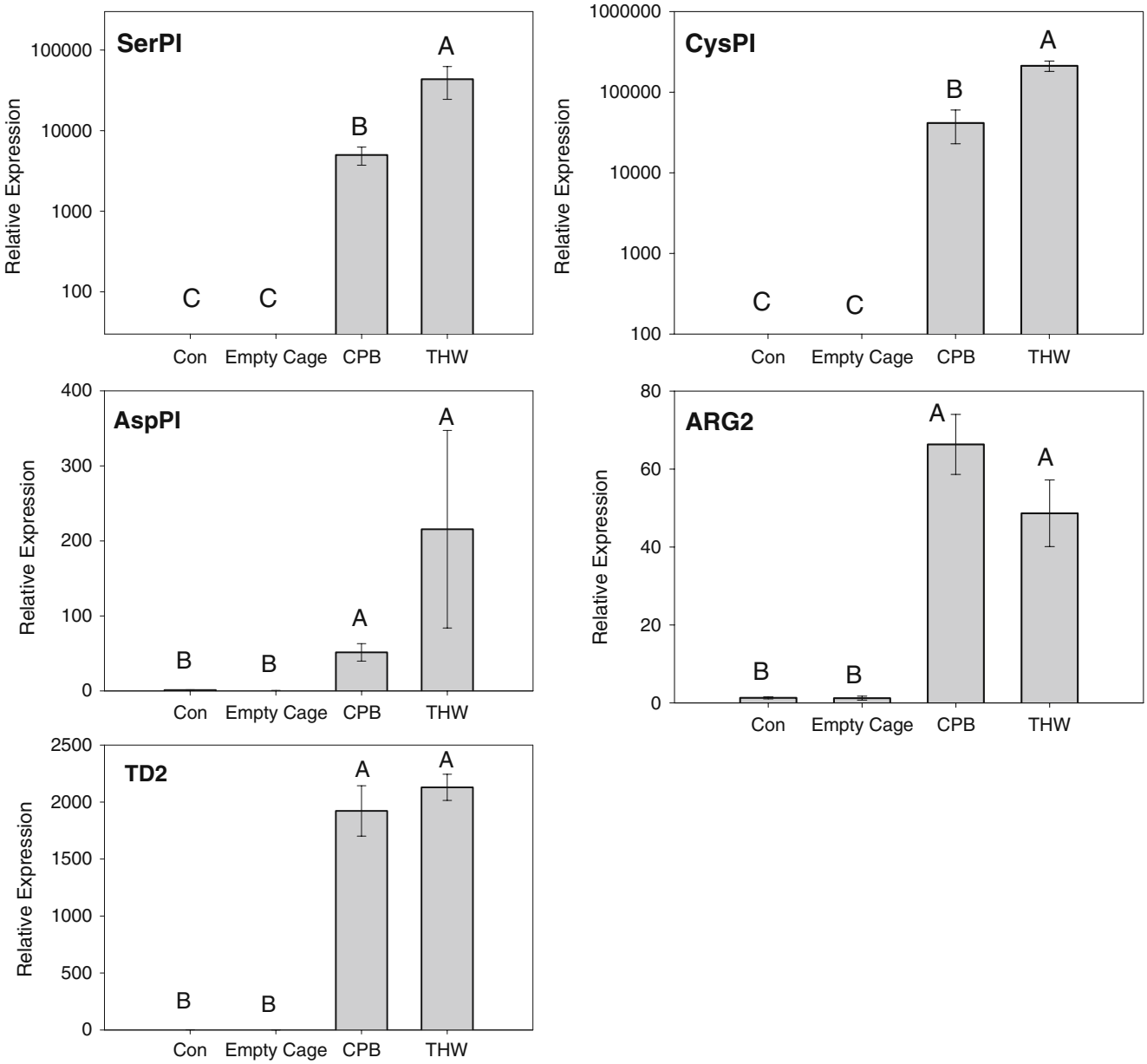


Fig. 1 Quantitative effects of insect feeding on expression of defense genes. Expression relative to ubiquitin was measured after 24 hr of Colorado potato beetle (CPB) or tobacco hornworm (THW) feeding (N=5). Con, unwounded control plants; Empty cages, unwounded plants with empty cages. *SerPI*, Serine protease inhibitor; *CysPI*, Cysteine protease inhibitor; *AspPI*, Aspartic protease inhibitor; *ARG2*, Arginase 2; *TD2*, Threonine deaminase 2. Values are mean ± SE. Different letters represent significant differences (Kruskal-Wallis; P<0.05)

P< 0.05). The amount of damage by the two herbivores was not significantly different (CPB consumption: 0.44±0.13 cm^2 and THW consumption: 0.81±0.15 cm^2; $P>0.05$). There were no significant correlations between the amount of damage by either CPB or THW and transcript accumulations (for CPB, *SerPI*: $r=0.346$, $P>0.05$, $N=5$; *CysPI*: $r=0.557$, $P>0.05$, $N=5$, for TWH, *SerPI*: $r=0.702$, $P>0.05$, $N=5$; *CysPI*: $r=0.476$, $P>0.05$, $N=5$). *SerPI* transcript accumulation was 9 fold higher in plants damaged by THW than in plants damaged by CPB (Fig. 1; $P<0.05$). *CysPI* transcript accumulation was 5 fold higher in plants damaged by THW than in plants damaged by CPB (Fig. 1; $P<0.05$). However, expression levels of *AspPI*, *ARG2*, and *TD2* were not significantly different in plants damaged by THW than in plants damaged by CPB (Fig. 1; $P>0.05$).

To investigate the effects of CPB and TWH feeding on trypsin PI activity, one larva of either CPB or THW was allowed to feed for 24 hr, followed by tissue extraction and assessment of trypsin PI activity 48 hr later. CPB and THW feeding induced higher trypsin PI activities than the control treatment (Fig. 2; $P<0.05$). Trypsin PI activities were 3-fold higher in plants damaged by THW than in plants damaged by CPB (Fig. 2; $P<0.05$).

Oral Secretions from CPB and THW Expression levels of defense genes were measured 12 hr after application of oral secretions to wounded leaves. Levels of defense gene transcripts were higher in wounded plants than in control plants (Fig. 3; $P<0.05$). Compared to mechanical wounding without oral secretions, CPB secretion suppressed expression levels of some defense genes, while THW secretion induced expression of defense genes (Fig. 3). Transcript accumulations of *CysPI*, *ARG2*, and *TD2* were 50% lower on wounded plants treated with CPB secretion than on control wounded plants (Fig. 3; *P< 0.05*). Expression levels of *SerPI*, *CysPI,* and *TD2* were 2- to 3-fold higher on wounded plants treated with THW secretion than on plants that were only wounded (Fig. 3; *P< 0.05*).

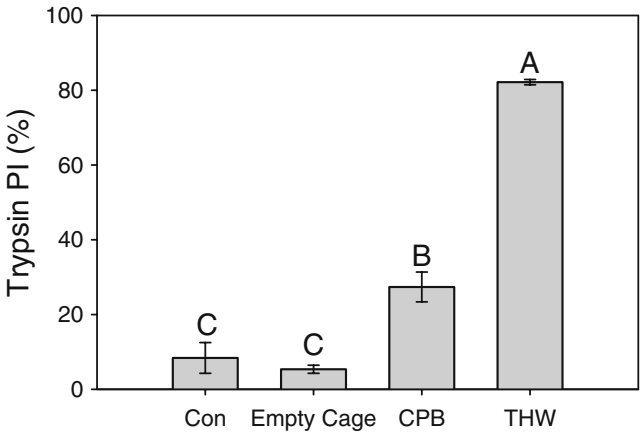


Fig. 2 Effects of insect feeding on trypsin proteinase inhibitor (PI) activity. Colorado potato beetle (CPB) or tobacco hornworm (THW) ($N=4$–6) were allowed to feed for 24 hr, followed by a 48 hr lag before activity was measured. Con, unwounded control plants; Empty cages, unwounded plants with empty cages. Values are mean ± SE. Different letters represent significant differences (Kruskal-Wallis; $P<0.05$)

CPB Growth Rate and PPO Activity CPB larvae grew more slowly when consuming leaf tissue that had previously been attacked by CPB or THW compared with consumption of control tissue (Fig. 4A; $P<0.05$). CPB growth on plants previously damaged by CPB was not significantly different from that on plants previously damaged by THW (Fig. 4A; $P>0.05$). PPO activity was measured 4 days after initiating insect feeding. CPB and THW feeding increased PPO activities compared with control treatment and PPO activities were higher on plants damaged by THW than on plants damaged by CPB (Fig. 4B; $P<0.05$).

Discussion

Coleopteran and Lepidopteran species frequently have different midgut pH levels and may use different proteases to digest plant proteins (Wolfson and Murdock, 1987; Felton and Duffey, 1991; Felton et al., 1992). The functions of induced antinutritive plant proteins such as PIs, ARG, TD, and PPO might vary depending on herbivore midgut pH and digestive enzymes. We hypothesized that tomato plants would respond specifically to different herbivores by increasing transcript accumulation of defense genes that are specifically effective against each herbivore. However, tomato did not express defense transcripts that encoded specific detrimental proteins against THW or CPB. Wounding by insect feeding or by mechanical damage increased transcript level for antinutritive proteins such as PIs, ARG, and TD, and increased activity for trypsin PI and PPO (Figs. 1, 2, 3). This is consistent with some other studies; for example, CPB feeding induced SerPI and PPO in tomato (Green and Ryan, 1972; Felton et al., 1992). Mechanical wounding, jasmonic acid, and herbivory all elicit the production of defense gene transcripts and proteins in tomato and potato leaves (Green and Ryan, 1972; Felton et al., 1992; Bolter, 1993; Korth and Dixon, 1997; Ryan, 2000; Wu and Haard, 2002).

Although *qualitative* differences in the induction of antinutritive proteins were not found, the induced responses of tomato were *quantitatively* different for different stimuli, suggesting specificity of elicitation in tomato. The level of transcripts for *SerPI* and *CysPI* was higher in plants damaged by THW than in plants damaged by CPB

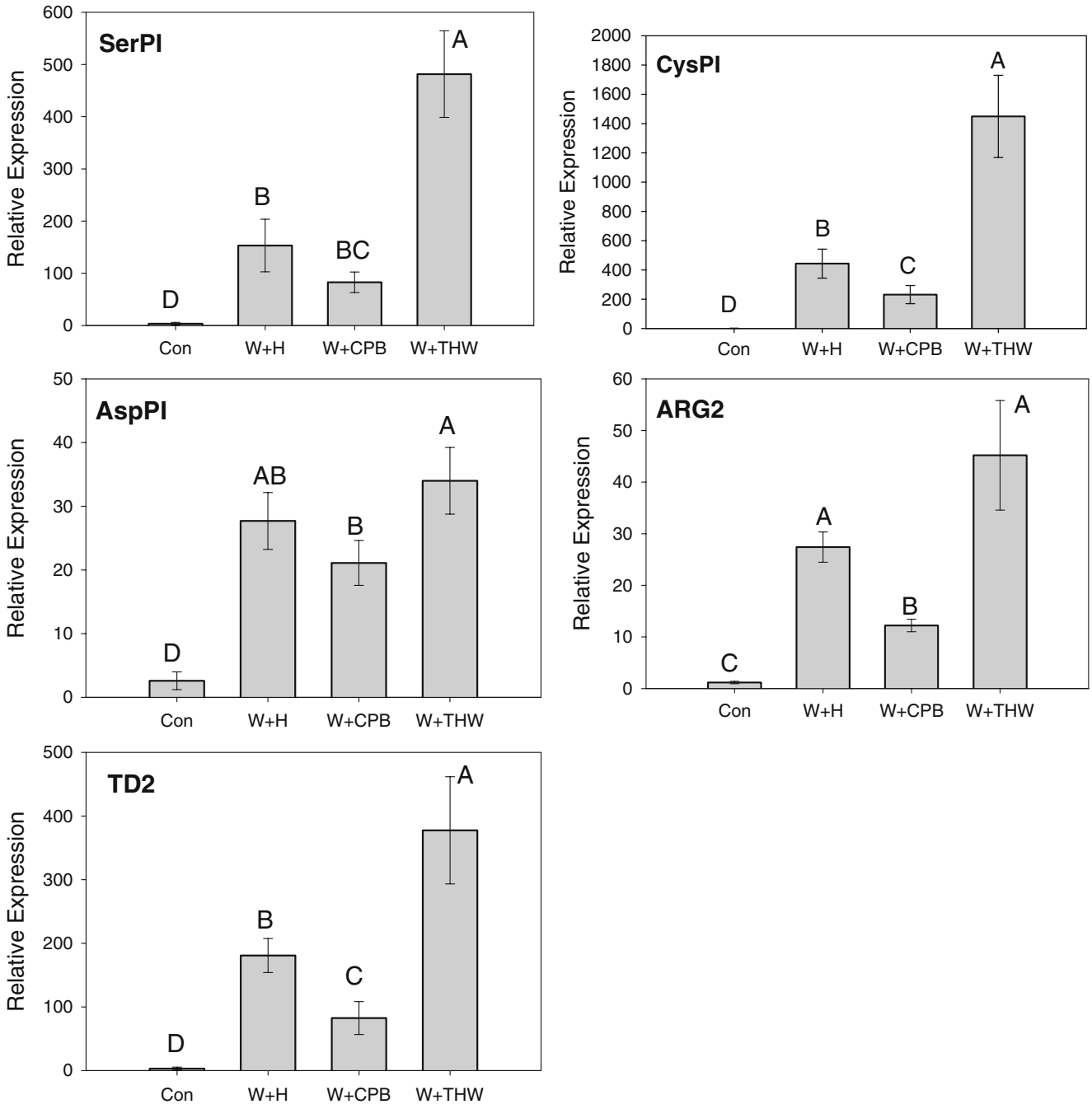


Fig. 3 Effects of oral secretions on expression of defense genes. Expression relative to ubiquitin was measured after 12 hr of treatment (N=5). Con, unwounded control plants; W+H, mechanical wounding plus H_2O; W+Colorado potato beetle (CPB), mechanical wounding plus CPB oral secretions; W+tobacco hornworm (THW), mechanical wounding plus THW oral secretions. *SerPI*, Serine protease inhibitor; *CysPI*, Cysteine protease inhibitor; *AspPI*, Aspartic protease inhibitor; *ARG2*, Arginase 2; *TD2*, Threonine deaminase 2. Values are mean ± SE. Different letters represent significant differences (Kruskal-Wallis; $P<0.05$)

(Fig. 1). Trypsin PI activity followed the same pattern as defense gene expression (Fig. 2). Traw and Dawson (2002) demonstrated specificity of elicitation in *Brassica nigra* in response to herbivores in the same feeding guild, but in different insect orders. Trichome densities and sinigrin concentration increased in plants damaged by a lepidopteran species, *Pieris rapae*, but did not change in plants damaged by the coleopteran species, *Phllotreata cruciferae*.

Few studies have investigated induced responses to herbivory by insects in different orders. However, species-specific induced responses elicited by different herbivores in the same feeding guild and insect orders have been noted

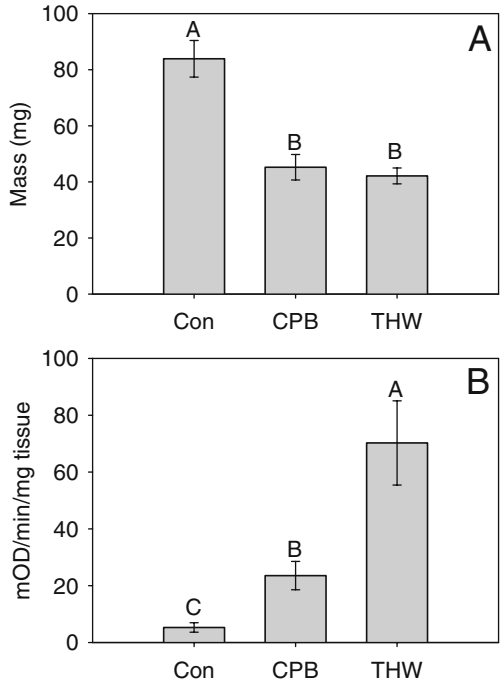


Fig. 4 Colorado potato beetle (CPB) growth and polyphenol oxidase (PPO) activity in tissue previously attacked by CPB or tobacco hornworm (THW) larvae. Plants were challenged with 5 CPB or 2 THW larvae for 24 hr. The damaged leaflets were excised 72 hr after removing the larvae, and one CPB larva was then allowed to feed for 6 days on the excised damaged leaves. (A) Mass of CPB larvae (N= 10–15). (B) PPO activity was measured from subsample of each treatment 4 days after initiating insect feeding (N=4). Con, unwounded control plants. Values are mean ± SE. Different letters represent significant differences (ANOVA; P<0.05)

in other studies. For example, in *Nicotiana attenuata*, transcriptional responses to a specialist *M. sexta* were different from those to the generalists *S. exigua* and *Heliothis virescens* (Voelckel and Baldwin, 2004). In *Boechera divaricarpa*, the specialist *Plutella xylostella* elicited transcription patterns different from the generalist *Trichoplusia ni* (Vogel et al., 2007). On the other hand, Reymond et al. (2004) reported that in *A. thaliana*, transcriptional responses to feeding by the specialist *P. rapae* or the generalist *S. littoralis* were similar. Thus, it appears that differential and specific induction may be found among some but not all systems of plants and herbivorous insects.

This study suggests that distinct responses to the herbivores may be due to insect-specific elicitors or effectors found in oral secretions, not due to differences in the amount of damage caused by different insects. The areas consumed by CPB and THW were not different, and there were no correlations between the amount of damage and transcript accumulation. We found that oral secretions from THW applied to mechanically wounded areas on tomato leaves induced transcription of defense genes, but oral secretions from CPB suppressed gene expression, except *SerPI* and *AspPI* (Fig. 3). In contrast, Lawrence et al. (2007) found that application of 100 µl of oral secretions from CPB to wounded tomato plants suppressed *SerPI* transcript accumulation 4 hr after the treatment, compared to wounded tomato plants. This discrepancy may be due to differences in the amount of oral secretion applied or to harvest time.

Oral secretions have been associated with herbivore-specific responses in several other systems. For example, oral secretion from THW increased production of jasmonic acid, transcription of defense-related genes, and release of volatiles in *N. attenuata* compared with the wounded control (Halitschke et al., 2001). Diezel et al. (2009) demonstrated that *M. sexta* and *S. exigua* feeding elicited different profiles of phytohormone production, perhaps due to differences in glucose oxidase activity and in fatty acid-amino acid conjugate profiles in the oral secretions of the two herbivore species. Bede et al. (2006) showed that glucose oxidase activity in saliva of *S. exigua* suppressed defense transcripts in *Medicago truncatula*. Lawrence et al. (2007) demonstrated that a protein fraction from CPB oral secretion may be responsible for suppression of PI transcript accumulation. In *N. attenuata*, oral secretion from THW suppressed nicotine accumulation (Winz and Baldwin, 2001). In *N. tabacum*, glucose oxidase (GOX) from *Heliocoverpa zea* saliva inhibited nicotine production (Musser et al., 2002). However, Schmelz et al. (2009) showed that tomato does not respond to elicitors such as volicitin (N-17-hydroxylinolenoyl-L -glutamine), N-linolenoyl-glutamine, inceptin, or califerin. In the current study, oral secretion from THW increased transcript accumulations of defense genes compared to wounded plants (Fig. 3). In *N. attenuata*, synthetic blends of N-linolenoyl-L-glutamine and N-linolenoyl-L-glutamate mimicked induced responses to oral secretion from THW. It is suggested that these two fatty acid-amino acid conjugates are necessary for herbivore-specific responses (Halitschke et al., 2003; Roda et al., 2004). Thus, it is likely that components other than fatty acid-amino acid conjugates in the oral secretion of THW might be responsible for the induced responses that we observed in tomato.

In tomato, application of oral secretions of CPB or THW to a wounded area on a leaf appears to mimic the induced responses to insect feeding. Peiffer and Felton (2009) showed that lepidopteran larvae release less than 10 nl of oral secretion, and may regurgitate during feeing on some host plant species. In that study, THW larvae did not regurgitate when they fed on tomato leaves, perhaps thus avoiding the induction of plant host defense responses. In contrast, CPB larvae consistently release oral secretion on tomato leaves (Chung and Felton, unpublished data), consistent with the suppression of plant defense responses by CPB oral secretion. We argue that CPB larvae secrete oral secretions that suppress host defense responses, but

THW larvae normally avoid secretion, thus minimizing the induction of defense responses.

The growth of CPB larvae was reduced to the same extent when the larvae consumed plants that had been previously damaged by either CPB or THW (Fig. 4), suggesting that there is low specificity of effect. The data also indicate that feeding by either species elicits the same kinds of antinutritive proteins. Although CPB feeding increased smaller amounts of defense proteins such as trypsin PI and PPO than THW feeding did, the levels might be sufficient to reduce subsequent CPB growth. In addition, it is possible that other defensive chemicals induced by CPB feeding deter CPB growth. Low specificity of effect has been found in other studies. Traw and Dawson (2002) showed that growth of *P. rapae* and *P. cruciferae* was reduced when the insects fed on plants damaged by either species. However, Agrawal (2000) showed a complex pattern for specificity of effect. In *Raphanus sativus*, three of four herbivore species showed reduced growth on plants damaged by conspecifics compared with undamaged control.

Overall, tomato shows distinct induced responses to different herbivores: induction by THW and minimal induction by CPB. Oral secretions of the two herbivores may play a key role in these species-specific responses. However, tomato does not increase transcripts that encode specifically deleterious proteins against THW or CPB when damaged by THW or CPB. There is low specificity of effect in tomato by CPB or THW feeding on subsequent CPB growth.

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Identification and Field Evaluation of Attractants for the Cranberry Weevil, *Anthonomus musculus* Say

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Abstract Studies were conducted to develop an attractant for the cranberry weevil, *Anthonomus musculus*, a pest of blueberry and cranberry flower buds and flowers in the northeastern United States. In previous studies, we showed that cinnamyl alcohol, the most abundant blueberry floral volatile, and the green leaf volatiles (*Z*)-3-hexenyl acetate and hexyl acetate, emitted from both flowers and flower buds, elicit strong antennal responses from *A. musculus*. Here, we found that cinnamyl alcohol did not increase capture of *A. musculus* adults on yellow sticky traps compared with unbaited controls; however, weevils were highly attracted to traps baited with the *Anthonomus eugenii* Cano aggregation pheromone, indicating that these congeners share common pheromone components. To identify the *A. musculus* aggregation pheromone, headspace volatiles were collected from adults feeding on blueberry or cranberry flower buds and analyzed by gas chromatography-mass spectrometry. Three male-specific compounds were identified: (*Z*)-2-(3,3-dimethyl-cyclohexylidene) ethanol (*Z* grandlure II); (*Z*)-(3,3-dimethylcyclohexylidene) acetaldehyde (grandlure III); and (*E*)-(3,3- dimethylcyclohexylidene) acetaldehyde (grandlure IV). A fourth component, (*E*)-3,7-dimethyl-2,6-octadien-1-ol (geraniol), was emitted in similar quantities by males and females. The emission rates of these volatiles were about 2.8, 1.8, 1.3, and 0.9 ng/adult/d, respectively. Field experiments in highbush blueberry (New Jersey) and cranberry (Massachusetts) examined the attraction of *A. musculus* to traps baited with the male-produced compounds and geraniol presented alone and combined with (*Z*)-3-hexenyl acetate and hexyl acetate, and to traps baited with the pheromones of *A. eugenii* and *A. grandis*. In both states and crops, traps baited with the *A. musculus* male-produced compounds attracted the highest number of adults. Addition of the green leaf volatiles did not affect *A. musculus* attraction to its pheromone but skewed the sex ratio of the captured adults towards females. Although the role of plant volatiles in host-plant location by *A. musculus* is still unclear, our studies provide the first identification of the primary *A. musculus* aggregation pheromone components that can be used to monitor this pest in blueberry and cranberry pest management programs.

Key Words Attractant · Blueberries · Coleoptera · Cranberries · Curculionidae · Gas chromatography-Mass spectrometry (GC-MS) · Grandlure · Host-plant volatiles · Monitoring · Pheromone · Semiochemical

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Introduction

The genus *Anthonomus* contains more than 100 North American species (Borror et al., 1989), many of which are considered among the most destructive pests of cultivated plants. One such example is the cranberry weevil, *Anthonomus musculus* Say (Coleoptera: Curculionidae), a pest that causes major economic losses annually to highbush blueberries, *Vaccinium corymbosum* L., and cranberries, *Vaccinium macrocarpon* Aiton (Ericales: Ericaceae), in the northeastern United States (Marucci, 1966; Averill and Sylvia, 1998; Long and Averill, 2003). Native to North America, *A. musculus* is distributed from Ontario (Canada) and New England (United States) to the Rocky Mountains and Florida (United States) (Lacroix, 1926; Averill and Sylvia, 1998). Although it utilizes many host plants within the family Ericaceae (Mechaber, 1992), *A. musculus* is considered a major pest only on cultivated *Vaccinium* spp. In New Jersey, this insect is commonly recognized as one of the top pests of highbush blueberries, whereas in Massachusetts it is considered a pest of cranberries (Doehlert and Tomlinson, 1947; Averill and Sylvia, 1998). *Anthonomus musculus* rarely occurs on cranberries in New Jersey (Mechaber, 1992).

Anthonomus musculus feeding and oviposition activity causes damage to blueberry and cranberry flower buds and flowers. Females lay their eggs inside unopened flowers, and the larvae complete their development within the flower buds. Most *A. musculus* adults overwinter outside commercial blueberry and cranberry fields, under debris and fallen leaves in surrounding wooded areas. Mechaber (1992) suggested that *A. musculus* can complete two generations a year, but a single generation is thought to be typical (Lacroix, 1926; Doehlert and Tomlinson, 1947). In New Jersey, adults become active in late April and move from overwintering sites to cultivated blueberries before the buds start to swell (Doehlert and Tomlinson, 1947). The adults emerge in late May–June (summer generation), and feed on tender leaves and young fruit. The majority of these adults eventually move out of the blueberry fields to alternative hosts in the surrounding woods, where they have been observed to mate (Mechaber, 1992; Z. Szendrei, personal observation). In Massachusetts, overwintering adults move from wild hosts (e.g., wild *Vaccinium* spp.) to cranberries in late May–June, mate, and lay eggs on the developing flower buds (Mechaber, 1992; Averill and Sylvia, 1998). Summer generation adults appear from late June to early July, and these adults will overwinter (Lacroix, 1926).

Current monitoring methods for *A. musculus* are labor-intensive and inaccurate. Because adults overwinter in wooded areas surrounding blueberry and cranberry fields, pest pressure is typically highest close to field edges; thus, monitoring efforts focus along these areas. Monitoring for adults is initiated when buds begin to swell. In highbush blueberries, overwintered *A. musculus* adults are monitored by using beating trays or by visually inspecting buds and flowers for injury; whereas in cranberries, adults are monitored by using sweep nets (Averill and Sylvia, 1998). In addition, adults are active almost exclusively on warm days. They drop to the ground when disturbed, and their populations are patchily distributed, causing inconsistent scouting results. Thus, a cost-effective and consistent sampling method for detecting *A. musculus* adults is critical to better estimate population occurrences and densities.

Previous studies have identified semiochemical-based attractants, i.e., aggregation pheromones, host-plant volatiles, and their combinations, which in some cases have been used successfully to monitor *Anthonomus* species (e.g., Tumlinson et al., 1969; Dickens, 1989; Eller et al., 1994; Innocenzi et al., 2001; Tinzaara et al., 2007). For example, Dickens (1989) showed that the green leaf alcohols (*E*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol, and 1-hexanol in combination with the boll weevil, *Anthonomus grandis* Boheman, aggregation pheromone enhances trap captures of boll weevils compared to the pheromone alone. Hence, our objective was to develop similar attractants to monitor more precisely *A. musculus* populations in blueberries and cranberries. In a recent study, we showed that female *A. musculus* are attracted to blueberry flowers, whereas males are attracted to undamaged flower buds and are repelled by damaged flower buds (Szendrei et al., 2009). In that study, volatile compounds from blueberry flower buds and flowers were identified; of these, cinnamyl alcohol and the green leaf volatiles (GLVs) (*Z*)-3-hexenyl acetate and hexyl acetate elicited strong antennal responses from both male and female *A. musculus*. Here, we conducted studies to: a) investigate the response of *A. musculus* to these blueberry volatiles and to the pepper weevil, *Anthonomus eugenii* Cano, and boll weevil, *A. grandis*, aggregation pheromones; b) isolate and identify the major components of the *A. musculus* aggregation pheromone; and c) evaluate the response of *A. musculus* to its aggregation pheromone alone and in combination with the antennally-active green leaf acetates.

Methods and Materials

Insects Field-collected *A. musculus* adults were used for headspace volatile collections. Adult *A. musculus* were collected in March–April 2008 and 2009 from highbush blueberries in Hammonton (Atlantic Co.) and Chatsworth (Burlington Co.), New Jersey, USA and transported to the laboratory. In the laboratory, adults were held in environmental chambers (16:8 hL:D, 15°C), in screen cages (30×30×30 cm), and were fed fresh highbush blueberry (cv. Bluecrop)

buds and foliage collected from an unsprayed field located at the P.E. Marucci Blueberry & Cranberry Center (Chatsworth, NJ, USA). Adult weevils were separated by sex under a stereomicroscope based on characters of the pygidium (Szendrei et al., 2009). The pygidium is fused with the last tergite in females, but these two segments are separate in males.

Volatile Collections Headspace volatiles were collected from *A. musculus* males and females in the laboratory (16:8 hL:D, 25°C), in 6 ml clear scintillation glass vials (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). The cap of each vial held an adsorbent trap, filled with 30 mg Super Q (80/100 mesh; Alltech, Deerfield, IL, USA), and a Pasteur pipette (Thermo Fisher Scientific Inc.), containing 1.25 g of activated charcoal (Alltech) to clean the incoming air. Volatiles from inside the vials were drawn through the trap by pulling air at a flow rate of ca. 1 l/min for 72 h. During volatile collections, weevils were provided daily with 0.3 g field collected blueberry or cranberry flower buds. Males and females were separated prior to volatile collections, and 20 adults of each sex were placed in a vial. In addition, volatile emissions were compared between males feeding on either blueberry or cranberry flower buds (N=4 replicates of 20 adults each per crop). After collections, traps were washed with 150 µl methylene chloride, an internal standard [400 ng of nonyl acetate (Sigma-Aldrich; St. Louis, MO, USA)] was added to each extract, and samples were stored in a freezer until used in gas chromatography-mass spectrometry (GC-MS) analysis.

Chemical Analyses Samples and synthetic standards were analyzed by GC-MS (Instruments 7890 and 5975 C; Agilent, Palo Alto, CA, USA) in both EI and CI (isobutane) modes by using a 30 m×0.25 mm i.d. × 0.25 µm film thickness medium polar DB35 (35%-Phenyl-methylpolysiloxane) and non-polar DB-1 (100% Dimethylpolysiloxane) capillary column (Agilent). Samples were introduced with cold on-column injection into a 1 m deactivated fused silica retention gap between injector and analytical column. For both columns, the oven temperature was kept at 30°C for 2.5 min after injection and then temperature programmed 20°C/min to 90°C and then 2°C/min to 125°C followed by 20°C /min to 240°C. The He carrier gas flow rate was 30 cm/sec (constant flow), and the MS transfer line temperature was 250°C. The ion source temperature was 220°C in EI mode and 250°C in CI mode. The presence of known pheromone components was confirmed by analyzing synthetic standards (compounds **1**–**7**; Fig. 1), as well as selected natural samples in EI- and CI-mode with both GC columns. For quantitative analyses on the DB-1 column, the synthetic standards and the internal standard nonyl acetate were analyzed in CI mode to establish diagnostic ions: for compounds **1**, **2**, **3**, and **6** they were *m/z* 155 (M + 1) and *m/z* 137 (M + 1-18); for compounds **4** and **5** it was *m/z* 153 (M + 1); for compound **7** it was *m/z* 169 (M + 1); and finally, for nonyl acetate it was *m/z* 187 (M + 1). The MS was set to collect data for all five ions during the whole analysis. In that way, known compounds could be selectively detected in the complex natural samples while at the same time making it possible to detect the presence of related compounds. Response factors for all compounds were calculated by injecting known amounts of the standards (for the synthetic geranic acid the areas for both isomers were added prior to conducting calculations).

Synthetic Chemicals (*Z*)-2-isopropenyl-1-methylcyclobutaneethanol (grandlure I; compound **1**, Fig. 1), (*Z*)-2-(3,3-dimethyl-cyclohexylidene)ethanol (*Z* grandlure II; compound **2**, Fig. 1), (*E*)-2-(3,3-dimethyl-cyclohexylidene) ethanol (*E* grandlure II; compound **3**, Fig. 1), a mixture of (*Z*)-(3,3-dimethylcyclohexylidene) acetaldehyde (grandlure III; compound **4**, Fig. 1) and (*E*)-(3,3- dimethylcyclohexylidene) acetaldehyde (grandlure IV; compound **5**, Fig. 1), (*E*)-3,7-dimethyl-2,6-octadien-1-ol) (geraniol; compound **6**, Fig. 1), and (*E*)-3,7-dimethyl-2,6-octadienoic acid [geranic acid (including neric acid); compound **7**, Fig. 1] were purchased from Bedoukian Research Inc. (Danbury, CT, USA) (all >95% purity). Cinnamyl alcohol, (*Z*)-3-hexenyl acetate, and hexyl acetate were purchased from Sigma-Aldrich (all≥97% purity).

2008 Field Experiments Field studies were conducted to test the response of *A. musculus* to yellow sticky traps (23 by 28 cm Unbaited Pherocon AM; Trécé Inc., Adair, OK,

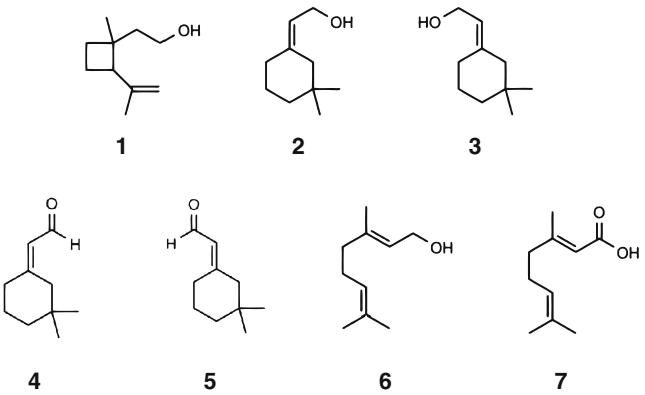


Fig. 1 Structures of seven common aggregation pheromone components in *Anthonomus* spp. weevils (Coleoptera: Curculionidae). **1**: (*Z*)-2-isopropenyl-1-methylcyclobutaneethanol (grandlure I); **2**: (*Z*)-2-(3,3-dimethyl-cyclohexylidene) ethanol (*Z* grandlure II); **3**: (*E*)-2-(3,3-dimethyl-cyclohexylidene) ethanol (*E* grandlure II); **4**: (*Z*)-(3,3-dimethylcyclohexylidene) acetaldehyde (grandlure III); **5**: (*E*)-(3,3-dimethylcyclohexylidene) acetaldehyde (grandlure IV); **6**: (*E*)-3,7-dimethyl-2,6-octadien-1-ol) (geraniol); and **7**: (*E*)-3,7-dimethyl-2,6-octadienoic acid (geranic acid)

USA) baited with cinnamyl alcohol and commercial *A. eugenii* pheromone (Trécé Inc.). Preliminary studies determined that *A. musculus* were caught successfully in yellow sticky traps placed at canopy level in highbush blueberry fields, whereas no weevils were caught in black cone traps placed on the ground for capturing walking insects (Z. Szendrei, unpublished data); thus, we used yellow sticky traps placed at canopy level in our studies. Cinnamyl alcohol is the main blueberry flower volatile (Szendrei et al., 2009), and was tested in three different types of release devices: plastic bubble (average release rate: 2.2 mg/d; ChemTica Internacional S.A., San José, Costa Rica), plastic 2-ml centrifuge vials (average release rate: 2 μg/day for 100 mg of cinnamyl alcohol in 1 ml mineral oil; ISCA Technologies, Riverside, CA, USA), and SPLAT® (Specialized Pheromone and Lure Application Technology, ISCA Technologies) at three different doses: 0, 30, 100 mg of cinnamyl alcohol per lure (mean release rate: 0.1 μg/d/mg lure). The responses to 10 treatments were compared to the response to the unbaited yellow sticky traps. All lures were attached to the sticky traps by using 22-gauge green florist wire. Traps were placed at four commercial highbush blueberry farms in New Jersey. One of the farms was located in New Lisbon (Burlington Co., NJ, USA), one in Chatsworth (Burlington Co., NJ, USA), and two in Hammonton (Atlantic Co., NJ, USA). Traps were positioned along the field edges near wooded borders, 10 m apart from each other, and were hung from a metal pole within the blueberry canopy ca. 1.6 m above ground. Treatments were set up in a randomized complete block design with four replications per farm. The position of the treatments within blocks was rotated weekly and the number of *A. musculus* on the traps was recorded weekly from April through June (11 wk). Lures were replaced with new ones every 3 wk.

2009 Field Experiments Field studies were conducted to test the attraction of adult *A. musculus* to a mixture of prospective components of the *A. musculus* aggregation pheromone at four commercial highbush blueberry farms in New Jersey (same locations as described above) and four cranberry farms in Massachusetts (three in East Wareham and one in West Wareham, Plymouth Co., MA, USA by using the same type of yellow sticky traps as in 2008. The *A. musculus* aggregation pheromone lures contained compounds **2**, **4**, **5**, and **6** loaded at a ratio of 4: 1: 1: 0.1, at a dose of 30 mg per lure, and a release rate of ca. 1 mg/day (ChemTica Internacional S.A., San José, Costa Rica). Because green leaf volatiles often synergize with insect pheromones (Reddy and Guerrero, 2004), we also tested the attraction of *A. musculus* to the GLVs (*Z*)-3-hexenyl acetate and hexyl acetate alone or in combination with the aggregation pheromone components. Both (*Z*)-3-hexenyl acetate and hexyl acetate are emitted from blueberry flower buds and flowers and elicit strong antennal responses from *A. musculus* (Szendrei et al., 2009). All custom-made lures were formulated into plastic bubble release devices (ChemTica Internacional S.A.) that were stapled to the yellow sticky traps. In addition to these lures, we also tested the commercially available *A. eugenii* and *A. grandis* pheromones (Trécé Inc.). Thus, we tested the following treatments: 1) geraniol (compound **6**); 2) (*Z*)-3-hexenyl acetate; 3) geraniol and (*Z*)-3-hexenyl acetate; 4) a blend of *Z* grandlure II (compound **2**), grandlure III (compound **4**), and grandlure IV (compound **5**); 5) a blend of *Z* grandlure II, grandlure III, grandlure IV, and geraniol (the *A. musculus* aggregation pheromone; see results); 6) a blend of *Z* grandlure II, grandlure III, grandlure IV, (*Z*)-3-hexenyl acetate and hexyl acetate; 7) a blend of *Z* grandlure II, grandlure III, grandlure IV, geraniol, and (*Z*)-3-hexenyl acetate; 8) the commercial *A. eugenii* pheromone lure; 9) the commercial *A. grandis* pheromone lure; and 10) an unbaited control. The GLV lures were loaded with 30 mg of (*Z*)-3-hexenyl acetate or hexyl acetate. Treatments were set up in a randomized complete block design with four replications at each of the eight farms. Treatments were rotated weekly among the positions in each block. In blueberries, traps were placed at canopy level as described above; and in cranberries traps were placed 1 m above canopy level. Traps were checked weekly between April and June (9 weeks) in blueberries and in May (3 weeks) in cranberries. Lures were replaced with new ones every 3 wk. All weevils captured on 27 April (overwintered adult generation) and 26 May (summer adults) in blueberry fields were removed from sticky traps and the sexes were separated.

2010 Field Experiments To further evaluate the *A. musculus* pheromone, an experiment was conducted to test the attraction of adult *A. musculus* to its aggregation pheromone in eight fields at four commercial highbush blueberry farms in New Jersey (same locations as described above). Yellow sticky traps were either baited with the ChemTica pheromone lures (containing compounds **2**, **4**, **5**, and **6** loaded at the ratio, dose, and release rate described above) or unbaited (controls). Trap position was the same as that described above, and the numbers of weevils on traps were recorded weekly from 1 April to 22 June.

Statistical Analyses For the 2008 and 2009 field data, the number of *A. musculus* captured in yellow sticky traps was analyzed by using a generalized linear mixed model (SAS Institute, Version 9.1, Cary, NC), with lure treatment as fixed factor, farm and block as random factors, and week as the repeated variable in the model. This analysis was used to determine the effects of lure types on attractiveness of traps to *A. musculus*. Differences among treatment means

were assessed by using the lsmeans statement with the pdiff option, and differences were considered significant at an α level of 0.05. Numbers of *A. musculus* were $\log_{10}$-transformed prior to analyses. Data were analyzed separately by years and states. For the 2010 data, a paired *t*-test was conducted to compare the number of *A. musculus* captured in pheromone-baited versus unbaited traps. We used a *Chi-square* test to determine whether there was a significant difference between the quantities of compounds emitted by male *A. musculus* feeding on cranberries versus blueberries and to test whether the sex ratio among captured weevils was different from 1:1 (α=0.05).

Results and Discussion

This study reports the first identification and field activity of components of the *A. musculus* aggregation pheromone. Our 2008 field experiment showed that there was a significant treatment effect on the number of *A. musculus* caught on yellow sticky traps among cinnamyl alcohol, *A. eugenii* aggregation pheromone, and unbaited control traps (F=3.80, df=9, 339, P<0.01) (Fig. 2). The *A. eugenii* pheromone-baited traps caught more *A. musculus* adults than all other treatments (t>3.12; df=9; P<0.01) (Fig. 2). These results are likely due to the fact that these two *Anthonomus* species share common pheromone components (Table 1). Subsequent headspace analysis of adult *A. musculus* showed that males emit (*Z*)-2-(3,3-dimethylcyclohexylidene) ethanol (*Z* grandlure II; compound **2**, Fig. 1), (*Z*)-(3,3-dimethylcyclohexylidene) acetaldehyde

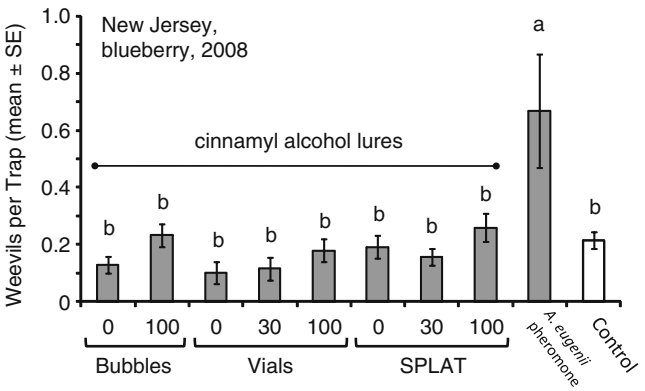


Fig. 2 Average number of *Anthonomus musculus* adults on yellow sticky traps baited with different lures at four commercial highbush blueberry farms in New Jersey in 2008. Cinnamyl alcohol was formulated in three different types of release devices at three different concentrations (numbers on x-axis represent cinnamyl alcohol concentrations in mg). The *Anthonomus eugenii* pheromone lure was purchased from a commercial source. Control yellow sticky traps were unbaited. Treatments with different letters above columns are significantly different (P≤0.05)

(grandlure III; compound **4**, Fig. 1), and (*E*)-(3,3-dimethylcyclohexylidene) acetaldehyde (grandlure IV; compound **5**, Fig. 1) (Fig. 3a). Female *A. musculus* did not emit any of these grandlure components (Fig. 3b); however, geraniol [(*E*)-3,7-dimethyl-2,6-octadien-1-ol; compound **6**, Fig. 1] was detected in similar quantities in male and female *A. musculus* samples when feeding on cranberry flower buds (Fig. 3b). This suggests that cranberry flower buds release geraniol in response to damage. When insects were fed blueberry buds, we did not detect geraniol in female samples (data not shown), but small quantities were found in male collections (Fig. 4), suggesting that this compound may be a part of the aggregation pheromone. Similarly, *A. eugenii* males emit compounds **2**, **4**, **5**, and **6**, but they also emit compounds **3** and **7** (Table 1; Eller et al., 1994). In the 1999 study, however, geraniol was emitted only by males; thus, all six compounds were designated as "male-specific" (Eller et al., 1994). So far, geraniol has been reported only from *A. eugenii* and *A. musculus* (Table 1), but it has been suggested as a possible host precursor for the different grandlures in *Anthonomus* spp. (Tumlinson et al., 1970). Compound **2** (the most abundant component of both *A. musculus* and *A. eugenii* pheromones; Table 1) as well as compounds **4** and **5** are also found in *A. grandis*; the strawberry blossom weevil, *Anthonomus rubi* Herbst. (Innocenzi et al., 2001); and the pecan weevil, *Curculio caryae* (Horn) (Hedin et al., 1997) (Table 1); however, compounds **4** and **5** were found in trace amounts in *A. rubi* and had no effect on their behavior (Innocenzi et al., 2001).

The emissions of the four *A. musculus* pheromone components: **2**, **4**, **5**, and **6** were 2.8±0.4 (mean±SE), 1.8±0.2, 1.3±0.2, and 0.9±0.1 ng/male/d, respectively. The total amount of all grandlures (i.e., sum of **2**, **4**, and **5**) emitted by male *A. musculus* was 5.6±0.7 (mean±SE) ng/male/d. This amount is >50-fold lower than those reported for *A. grandis* (4.2 μg/male/d; Chang et al., 1989), *A. eugenii* (15 μg/male/d; Eller et al., 1994), *A. rubi* (8.1 μg/male/d; Innocenzi et al., 2001), and *C. caryae* (0.3 μg/male/d; Hedin et al., 1997). Our quantitative assessment of the pheromone components may have been influenced by the field-collected weevils of unknown age and sexual maturity that we used in our assays. Once methods of maintaining these insects in laboratory culture have been worked out, we intend to control the developmental and reproductive variables to achieve a more precise determination of the quantity of pheromone components emitted.

There were minimal differences in volatile collections from *A. musculus* feeding on different host plants (blueberries versus cranberries). Qualitatively, the headspace of male *A. musculus* that were feeding on blueberry flower buds did not differ from those feeding on cranberry flower buds (Fig. 4). Quantitatively, grandlure components in the headspace of male *A. musculus* sampled on blueberry

Table 1 Comparison of the composition and relative abundance of the aggregation pheromone in grandlure-emitting weevil species[a]

Compound[b]	*Anthonomus musculus* (Cranberry Weevil)	*Anthonomus eugenii*[c] (Pepper Weevil)	*Anthonomus grandis*[d] (Boll Weevil)	*Anthonomus rubi*[e] (Strawberry Blossom Weevil)	*Curculio caryae*[f] (Pecan Weevil)
1			36	15	25
2	43	48	35	75	55
3		32		trace	
4	23	3	14	trace	10
5	20	2	15	trace	10
6	14	2			
7		13			
Lavandulol				10	

[a] Table entries are percentages of amounts

[b] Names of compounds are provided in Fig. 1

[c] Eller et al. (1994)

[d] Chang et al. (1989)

[e] Innocenzi et al. (2001)

[f] Hedin et al. (1997)

versus cranberry hosts were similar, but geraniol (compound **6**; Fig. 1) was significantly (χ^2=19.25, *df*=3, *P*< 0.01) more abundant in the headspace from males feeding on cranberries (Fig. 4). Although the reason for this difference remains unknown, it is possible that host plant might affect the production of geraniol in male *A. musculus* or its emission after herbivore feeding damage. Geraniol is a monoterpenoid alcohol commonly emitted from plants, particularly flowers (Schiestl, 2010), that is induced by herbivore feeding (Han and Chen, 2002), and with known activity on insects, including mosquitoes (e.g., Qualls and Xue 2009) and bees (e.g., Williams et al., 1981). Besides *Anthonomus* spp., it is a component of the honey bee pheromone (Boch, 1962; Pickett et al., 1980). Geraniol is a

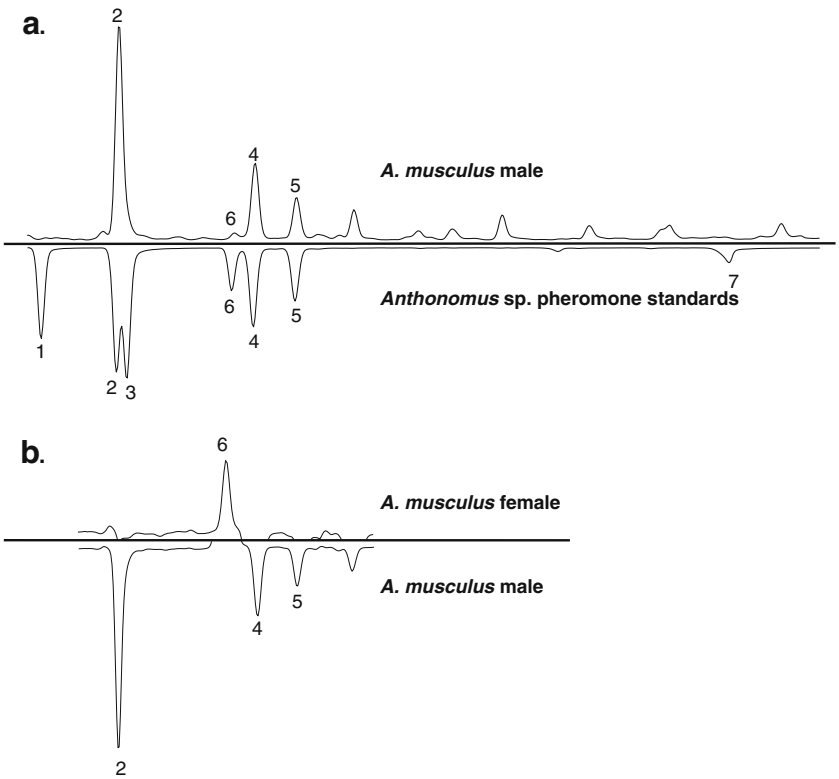


Fig. 3 Comparison between a representative chromatogram trace of male *Anthonomus musculus* (top) and a blend of standard pheromone components from *Anthonomus* spp. (bottom) **a**; and between male and female *A. musculus*, showing a selective ion trace of female samples when male compounds were subtracted from the trace (top) and for male samples when female peaks were subtracted (bottom) **b**. Names of compounds 1–7 are provided in Fig. 1

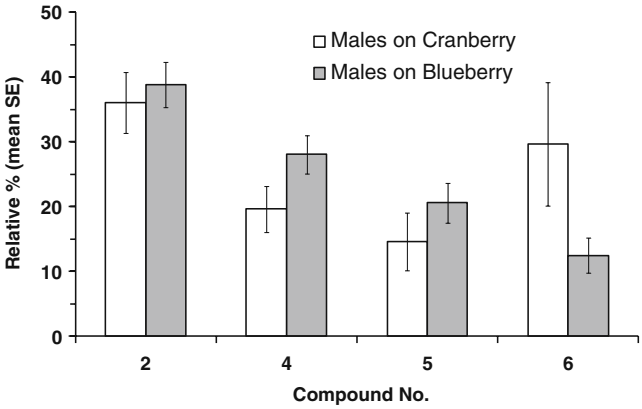


Fig. 4 Emissions of the four identified *Anthonomus musculus* aggregation pheromone components from males fed on blueberry and cranberry flower buds. Amounts of compound **6** (geraniol) were significantly greater in males fed on cranberry flower buds compared with those fed on blueberry flower buds ($P<0.05$); host plant did not affect any of the grandlure compounds **2**, **4**, and **5** ($P>0.05$). Names of compounds **2**, **4**, **5**, and **6** are provided in Fig. 1. $N=4$ replicates (of 20 males each) per crop

possible precursor of the *A. grandis* pheromone (Tumlinson et al., 1970; Thompson and Mitlin, 1979), and can be either derived from the host plant or produced *de novo* by male *Anthonomus* spp. Blueberry flower buds and flowers, however, did not emit detectable amounts of geraniol even after damage by *A. musculus* adults (Szendrei et al., 2009); thus, it is likely that *A. musculus* produces this compound. We will conduct more studies to elucidate the importance of host plant parts and phenology on pheromone production in *A. musculus*.

To support our behavioral data from field flight trapping (see below), we also attempted to evaluate headspace volatiles from *A. musculus* by gas chromatography-electroantennal detection (GC-EAD). However, this was a major technical challenge because of the small size of the insects and the relatively small amount of pheromone components in the extracts (see above). Similar problems have been encountered with the plum curculio, *Conotrachelus nenuphar* (Herbst) (Curculionidae) (T. Leskey and Aijun Zhang, USDA-ARS, Beltsville, MD, USA, personal correspondence). We plan to continue to pursue electrophysiological assays of this system in collaboration with Drs. Leskey and Zhang.

In 2009, the attractiveness of the identified main four-component pheromone blend to *A. musculus* was evaluated in commercial blueberry and cranberry farms. In New Jersey blueberry fields, the numbers of adult *A. musculus* caught on yellow sticky traps with different lure treatments were significantly different ($F=15.02$; df=9, 315; $P<0.01$) (Fig. 5a). Yellow sticky traps baited with compounds **2**, **4**, **5**, and **6** (*A. musculus* aggregation pheromone components) caught more adult *A. musculus* ($t>2.21$; $df=9$; $P<0.03$) than any of the other lure treatments. Relative to the unbaited control, this treatment attracted ~2.9 times more weevils over the course of the field season. All treatments that contained the *A. musculus* species-specific lure increased attraction of weevils to traps relative to the unbaited control, lures

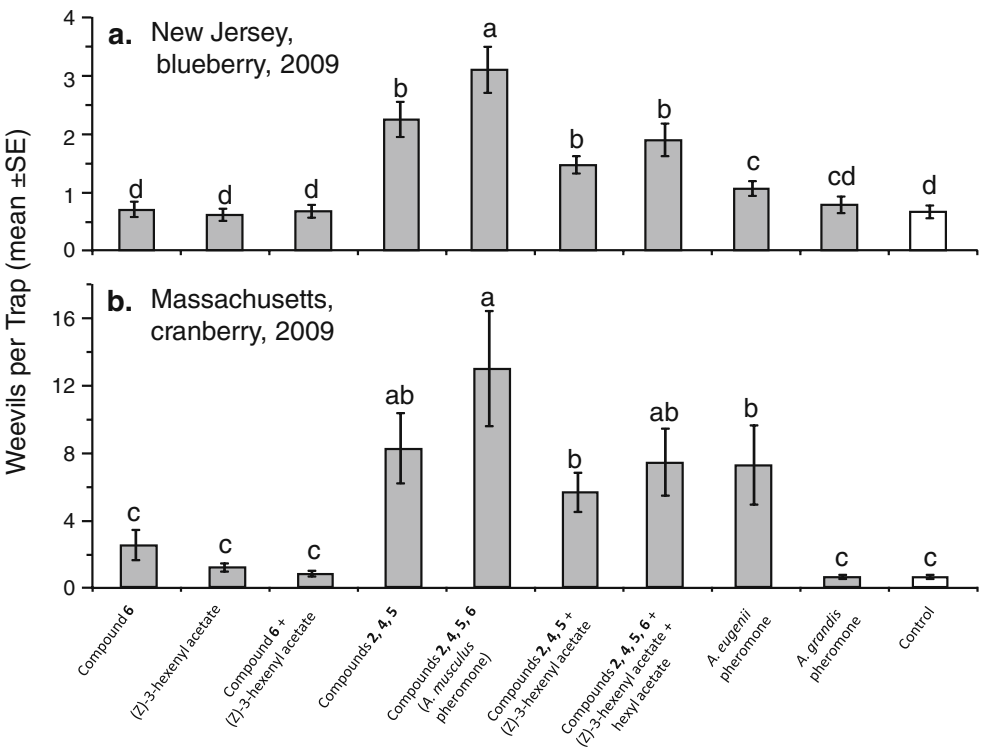


Fig. 5 Mean (± SEM) trap catch of *Anthonomus musculus* on yellow sticky traps baited with different compounds in the 2009 field experiment in New Jersey **a** and Massachusetts **b**. Names of compounds **2**, **4**, **5**, and **6** are provided in Fig. 1. Treatments with different letters above bars are statistically different ($P\leq0.05$)

containing only plant volatiles, and related *Anthonomus* species pheromones (t>3.39; df=9; P<0.05). Although the number of weevils in blueberry fields was lower in 2010 than in 2009, our results from 2009 were confirmed in 2010: i.e., ~ 2 times more adult *A. musculus* were captured in pheromone-baited compared with unbaited traps (mean number of weevils (± SE) in: unbaited traps=4.9 ±1.3; pheromone-baited traps=9.3±1.7; t=2.57, P=0.037). Lures baited with the *A. musculus* pheromone (compounds **2**, **4**, **5**, and **6**) caught similar numbers of males and females in blueberry fields (Table 2). However, females from the summer generation but not from the overwintered generation were significantly more attracted to lures baited with compounds **2**, **4**, and **5** (Table 2), indicative that newly emerged, virgin females might be more responsive to the male-produced grandlures. Similar variation in male:female ratio in response to pheromone-baited traps was observed for *A. rubi* during the trapping season (Innocenzi et al., 2001).

In Massachusetts cranberries, the numbers of *A. musculus* caught on yellow sticky traps were also different among lure treatments (F=9.02; df=9, 90; P<0.01), (Fig. 5b). The most attractive trap for *A. musculus* weevils in this state and crop was the same as in New Jersey, i.e., traps baited with the lure containing compounds **2**, **4**, **5**, and **6**. The response of *A. musculus* weevils to the other *Anthonomus* pheromones was similar in the two states and crops (Fig. 5). Compared to the unbaited control, the *A. eugenii* pheromone but not the *A. grandis* pheromone attracted more *A. musculus* weevils. Four of six pheromone components are shared by *A. musculus* and *A. eugenii*; whereas, three of four components are shared between *A. musculus* and *A. grandis* (Table 1). Key differences are the presence of compound **1** in the *A grandis* pheromone blend and compounds **3** and **7** in the *A. eugenii* blend; these compounds are absent in the *A. musculus* blend and can account at least in part for the specificity in *A. musculus* attraction to its aggregation pheromone. Overall, the abundance of weevils in yellow sticky traps was approximately 4 times higher in Massachusetts than in New Jersey.

In our 2009 and 2010 field studies, 30 mg of compounds **2**, **4**, **5**, and **6** were loaded at a 4:1:1:0.1 ratio. This ratio was estimated based on preliminary data collected from a few males. Subsequent analysis (Table 1; Fig. 4) with more replications revealed that the ratio of these compounds is closer to 2:1:1:0.7. Further studies are needed to determine whether this latter ratio is more attractive to *A. musculus*. Despite using a potentially suboptimal ratio, our field study clearly shows that a lure containing compounds **2**, **4**, **5**, and **6** attracts adult *A. musculus*. Whether all components in the *A. musculus* pheromone blend are needed for this attraction requires further investigation; however, a blend of compounds **2**, **4**, and **5** was less attractive than the full blend, and compound **6** alone did not attract *A. musculus* (Fig. 5). Most recent analysis of the headspace emissions from adult *A. musculus* indicate the presence of two previously undetected minor compounds (an acid similar to nerolic/geranic acid and an alcohol similar to nerol/geraniol; data not shown) released by males only, which might have behavioral significance. Future studies will identify these compounds and determine their biological activity.

Table 2 Trap catch and sex ratio of male and female *Anthonomus musculus* captured in response to synthetic lures in New Jersey blueberry fields

Lure[a]	27 April 2009 (Overwintered Adults)			26 May 2009 (Summer Generation Adults)			Totals (Both Generations)		
	Female	Male	Ratio	Female	Male	Ratio	Female	Male	Ratio
Control	16	15	1.1	2	5	0.4	18	20	0.9
Anthonomus grandis pheromone	16	26	0.6	2	2	1.0	18	28	0.6
Anthonomus eugenii pheromone	23	21	1.1	8	15	0.5	31	36	0.9
Compounds **2**, **4**, **5**, **6** + (*Z*)-3-hexenyl acetate + hexyl acetate	30	20	1.5	55	26	2.1**	85	46	1.8**
Compounds **2**, **4**, **5** + (*Z*)-3-hexenyl acetate	23	13	1.8	40	7	5.7**	63	20	3.2**
Compounds **2**, **4**, **5**, **6** (*Anthonomus musculus* pheromone)	30	18	1.7	85	83	1.0	115	101	1.1
Compounds **2**, **4**, **5**	30	27	1.1	57	34	1.7*	87	61	1.4*
Compound **6** + (*Z*)-3-hexenyl acetate	20	20	1.0	4	4	1.0	24	24	1.0
(*Z*)-3-hexenyl acetate	12	15	0.8	5	0		17	15	1.1
Compound **6**	15	12	1.3	10	4	2.5	25	16	1.6

[a] Names of compounds 2, 4, 5, and 6 are provided in Fig. 1

* $0.05 \geq P \geq 0.01$

** $P \leq 0.01$

In addition to the aggregation pheromone, volatiles from host plants may inform foraging *A. musculus* about suitable feeding and oviposition sites. Several species related to *A. musculus*, e.g., *Anthonomis pomorum*, *A. grandis*, and *A. rubi*, have shown electrophysiological antennal (EAG) responses to host plant volatiles (Dickens, 1990; Kalinova et al., 2000; Bichao et al., 2005) and several species of weevils, such as the pine weevil, banana weevil, vine weevil, and pepper weevil (Budenberg et al., 1993; Wibe et al., 1997; van Tol and Visser, 2002; Addesso and McAuslane, 2009), responded to host-plant volatiles in behavioral bioassays. In Y-tube olfactometer assays, Mechaber (1992) found that adult *A. musculus* were more attracted to conspecific-damaged cranberry vines compared with undamaged vines, as well as to undamaged and damaged flower buds compared to clean air (Mechaber, 1992). Recently, we found that female *A. musculus* are attracted to blueberry flowers, whereas males are attracted to undamaged flower buds and are repelled by damaged flower buds (Szendrei et al., 2009). These studies indicate that *A. musculus* adults might use host-plant volatiles during host or mate location, but did not identify the specific volatile cues responsible for this attraction. In this study, we tested two potential host-plant attractants for *A. musculus*. In 2008, we tested the main blueberry floral component, cinnamyl alcohol, at different doses and formulations. However, regardless of the dose and formulation, cinnamyl alcohol baited traps caught similar numbers of *A. musculus* when compared to the control trap (Fig. 2). In 2009, we tested the GLV (*Z*)-3-hexenyl acetate. GLV compounds are highly ubiquitous in plants and play important roles in insect-plant interactions (Visser, 1986; Szendrei and Rodriguez-Saona, 2010). For example, a mixture of plant volatiles that includes (*Z*)-3-hexenyl acetate attracts the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Visser, 1986), and the scarab beetle *Anomala octiescostata* Burmeister (Leal et al., 1994). Although (*Z*)-3-hexenyl acetate was found in headspace collections from both blueberry flower buds and flowers and elicited significant EAG responses in *A. musculus* (Szendrei et al., 2009), it did not increase adult weevil attraction compared with unbaited control traps (Fig. 5). Host-plant volatiles, particularly GLVs, can enhance the response of insects to their pheromone (Dickens et al., 1990; Reddy and Guerrero, 2004). For example, (*Z*)-3-hexenyl acetate enhanced the response of the diamondback moth, *Plutella xyllostella* (L.), to its sex pheromone (Reddy and Guerrero, 2000). A synergistic effect has also been reported between GLVs and the *A. grandis* aggregation pheromone (e.g., Dickens, 1989). Here, we tested the response of *A. musculus* to its aggregation pheromone in combination with (*Z*)-3-hexenyl acetate and hexyl acetate. Our results suggest an interruptive effect of these GLVs: addition of (*Z*)-3-hexenyl acetate to a blend of compounds **2**, **4**, and **5** reduced numerically (but not significantly) *A. musculus* trap captures (Fig. 5). Similarly, addition of (*Z*)-3-hexenyl acetate and hexyl acetate to a blend of compounds **2**, **4**, **5**, and **6** reduced (significantly in New Jersey and numerically in Massachusetts) *A. musculus* trap captures (Fig. 5). A reduction in the number of insects caught in traps was observed when the GLVs (*E*)-2-hexen-1-ol, (*Z*)-2-hexen-1-ol, and (*Z*)-3-hexen-1-ol were added to lineatin, the aggregation pheromone of the striped ambrosia beetle, *Trypodendron lineatum* Olivier (Borden et al., 1997), as well as when the GLVs 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-3-hexen-1-ol were added to the pheromone of the spruce bark beetle, *Ips typographus* (L.) (Zhang et al. 1999). Furthermore, addition of the GLV acetates to the *A. musculus* aggregation pheromone skewed the sex ratio of the captured weevils towards females in the summer (Table 2).

Similarly to our results, Cha et al. (2008) failed to find a significant change in the number of male grape berry moths captured between host-plant volatile-baited and control traps. Plant volatile attractants for codling moth failed under certain cases in the field (Knight and Light, 2005). The failure of plant volatiles to attract insects to traps in our field experiments could be because the tested volatiles were already present in the environment at the time when lures were placed in the field, and the background cues may have masked odors from the lures (Schröder and Hilker, 2008). In the case of cinnamyl alcohol, habituation or lack of sensitivity may also explain the low response rate, since this volatile stands out prominently from the background during bloom in blueberry fields. It is also possible that male *A. musculus* were repelled by the GLV plus pheromone baits as a result of odor camouflaging, where an odor is less detectable when presented in a combination with other odors. In our case, the quality or quantity of GLVs may have disrupted the perception of pheromone compounds by the insect and thus lead to lower or non-significant response.

Although the role of plant volatiles in host-plant location by *A. musculus* remains unclear, the *A. musculus* aggregation pheromone identified here can be an easy-to-use and cost-effective tool for monitoring this pest in blueberry and cranberry pest management programs. In highbush blueberries, an attractant for this weevil will be most useful for early detection of overwintered adults. The *A. musculus* pheromone was more attractive to the newly-emerged summer generation adults than to the overwintered adults (Table 2). Considering that the overwintered adults are the most damaging to the crop, the pheromone needs to be optimized by testing different pheromone doses, formulations, and ratios. Then, traps must be optimized by testing, trap colors, designs, and placements. A refined pheromone

blend that may contain plant volatiles and other minor components is required to optimally monitor *A. musculus* populations. Once optimized, this pheromone can also be used to develop an attract-and-kill formulation to manage *A. musculus*, similarly to the ones tested for *A. grandis* (e.g., McKibben et al., 1990).

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Identification and Field Evaluation of the Sex Pheromone of *Synanthedon bicingulata* (Staudinger)

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Abstract The sex pheromone of *Synanthedon bicingulata* (Staudinger), a major pest of *Prunus* species in many regions of northeast Asia, was identified. Two major components from the pheromone gland extracts of female moths are (*E*,*Z*)-3,13-octadecadienyl acetate (*E*3,*Z*13-18:OAc) and (*Z*,*Z*)-3,13-octadecadienyl acetate (*Z*3,*Z*13-18:OAc), and the average ratio of these components is about 4:6, respectively. In addition to the major components, four minor components, (*Z*)-13-octadecenyl acetate (*Z*13-18:OAc), (*E*,*Z*)-2,13-octadecadienyl acetate (*E*2,*Z*13-18:OAc), (*E*,*Z*)-3,13-octadecadien-1-ol (*E*3,*Z*13-18:OH), and (*Z*,*Z*)-3,13-octadecadien-1-ol (*Z*3,*Z*13-18:OH) also were identified from pheromone gland extracts. Field tests showed that *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc are essential for attraction of male *S. bicingulata* moths, and males are optimally attracted to the blend ratio found in pheromone gland extracts of conspecific females. Addition of the minor glandular components (*Z*13-18:OAc, *E*2,*Z*13-18:OAc, *E*3,*Z*13-18:OH, and *Z*3,*Z*13-18:OH) did not affect captures of males to the primary binary blend. Thus, the blend of *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc at the natural ratio can be used for monitoring populations of this species.

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Key Words *Synanthedon bicingulata* · (*E*,*Z*)-3,13-octadecadienyl acetate · (*Z*,*Z*)-3,13-octadecadienyl acetate · Lepidoptera · Sesiidae

Introduction

Synanthedon bicingulata (Staudinger) (Lepidoptera: Sesiidae) is an economically important pest of *Prunus* species (Rosaceae), such as *P. persica*, *P. mume*, *P. salicina*, and *P. serrulatavar* in Korea, China, and the Russian Far East (Arita et al., 2004). Larvae feed on the cambial tissue within tree trunks of host plants, resulting in significant production losses. This insect can be difficult to control with conventional insecticides due to the sheltered larval development. Species-specific sex pheromone baited traps for *S. bicingulata* may be useful for monitoring populations and timing application of control measures. In addition, the pheromone is potentially useful for population control using mating disruption or mass trapping (e.g., Leskey et al., 2009). The sex pheromone of *S. bicingulata* has not been previously reported.

Although sex attractants for more than 110 sesiid moth species have been determined in field screening experiments, to date, sex pheromones of only 19 species have been identified (El-Sayed, 2010). Typically, sesiid moths use 3,13- and 2,13-octadecadien-1-ols and their corresponding acetates and aldehydes as sex pheromone components. In moths, species specificity of sex pheromone blends is responsible for pre-mating reproductive isolation between sympatric species (Löfstedt et al., 1991; Yang et al., 2009a). Therefore, it is important to examine the pheromone components produced by different species to understand the evolutionary diversification of pheromone signals in Lepidoptera (Roelofs and Brown, 1982). We report here the

chemical identification and field testing of the female-produced sex pheromone blend of *S. bicingulata*.

Methods and Materials

Insects Pupae of *S. bicingulata* were collected by excavating them from infested trunks of peach trees in May 2009 at Jeonju (35.4°N,127.0°E), Korea. They were placed individually into capped plastic cups (30 ml), and maintained at 25°C under a 14L:10D photoperiod. After emergence, the sexes were separated based on distinctive hair tufts. Moths were provided with a cotton pad soaked with a 10% sucrose solution as food.

Chemicals Synthetic pheromone standards used in this study were purchased from Pherobank (Wageningen, The Netherlands). Isomeric purity of these compounds exceeded 99%. Straight-chain hydrocarbons, dimethyldisulfide, iodine, and sodium thiosulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Pheromone Extraction Virgin females of *S. bicingulata* exhibited the characteristic calling posture by extruding the abdominal tip during photophase. Therefore, pheromone gland extracts were taken from 1- to 2-d-old females at 7 h into the photophase. Excised pheromone glands were placed in 10 μl hexane in a 0.3-ml conical glass vial (Wheaton, Millville, NJ, USA) for 10 min at room temperature. The supernatant was transferred to a clean vial and stored at −20°C until analysis.

Dimethyldisulfide Derivatizations Dimethyldisulfide (DMDS) derivatives of pheromone gland extracts and synthetic standards were prepared according to procedures by Buser et al. (1983). Approximately 50 μl DMDS and 5 μl iodine solution (60 mg of I_2 in 1 ml of diethyl ether) were added to an extract (10 μl) of 18 female equivalents of *S. bicingulata* pheromone, and held at 60°C for 48 h. After addition of 50 μl of sodium thiosulfate (5%), the organic layer was transferred to a clean vial and concentrated to ca. 10 μl for GC-MS analysis. The same procedures described above were followed for hexane solutions (10 ng in 10 μl n-hexane) of synthetic *Z*3-18:OAc, *E*3,*Z*13-18:OAc, *Z*3,*Z*13-18:OAc, *E*2,*Z*13-18:OAc, *E*3,*Z*13-18:OH, and *Z*3,*Z*13-18:OH.

Chemical Analysis Pheromone gland extracts and synthetic standards were analyzed on an Agilent 6890N GC interfaced to an Agilent 5975C mass-selective detector. Samples were run on DB-Wax and DB-23 columns (30 m×0.25 mm ID, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). The oven temperature was maintained at 80°C for 1 min, increased to 180°C at 10°C/min, then to 220°C at 5°C/min, and held at the final temperature for 10 min. Helium was the carrier gas (1 ml/min), and injections were splitless. Injector temperature was 250°C with the purge valve opening 0.75 min after a manual injection. The ionization voltage was 70 eV, and the ion source temperature was 230°C. Compounds from pheromone gland extracts were identified tentatively by comparison of their mass spectra with the mass spectra library (Wiley-NIST, Hoboken, NJ, USA); and identifications were subsequently confirmed by comparison of retention indices (RI; relative to alkane standards, Van den Dool and Kratz, 1963), and mass spectra with those of authentic standards on two different columns. DMDS derivatives were analyzed with a DB-1 column (30 m× 0.25 mm ID, 0.25 μm film thickness), using a temperature program of 100°C to 300°C at 10°C/min. The GC conditions were the same as those described above.

Field Experiments Experiments were conducted in June 2009 at Jeonju, Korea. Sticky delta traps (Green Agro Tech, Korea) baited with rubber septa (Aldrich Chemical Co., Milwaukee, WI, USA) impregnated with candidate pheromone components in hexane were hung in peach orchards at a height of 1.5 m. Field tests employed a complete randomized block design with four replicate blocks of each treatment. The distance between traps within a block was about 10 m. Captured moths were counted at intervals of 3–4 d.

Experiment 1 compared attraction of male *S. bicingulata* to *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc alone or in various combinations. Experiment 2 tested whether attractiveness of standard baits of two major components could be enhanced by addition of four minor components identified in female gland extracts; *Z*13-18:OAc, *E*2,*Z*13-18:OAc, *E*3,*Z*13-18:OH, and *Z*3,*Z*13-18:OH. Trap catch data (x) were transformed to log (x+1) and submitted to one-way analysis of variance (ANOVA). Treatments that failed to capture males were not included in the analyses to avoid violating assumptions of ANOVA. Means were compared by Tukey's test at α=0.05 (SAS Institute Inc. 2008).

Results

Chemical Analysis Analysis of pheromone gland extracts of female *S. bicingulata* by GC-MS revealed the presence of two major components and four minor components, as well as normal alkanes including pentacosane and heptacosane (Fig. 1). The mass spectrum of compound 1 showed a molecular ion *m/z* 310, and diagnostic fragment ions *m/z* 250 (M-60), 222, and 61 (indicative of an acetate functionality), which suggested an octadecenyl acetate. The DMDS adduct of compound 1 showed a molecular ion *m/z* 404 and diagnostic ions *m/z* 287, 227, and 117,

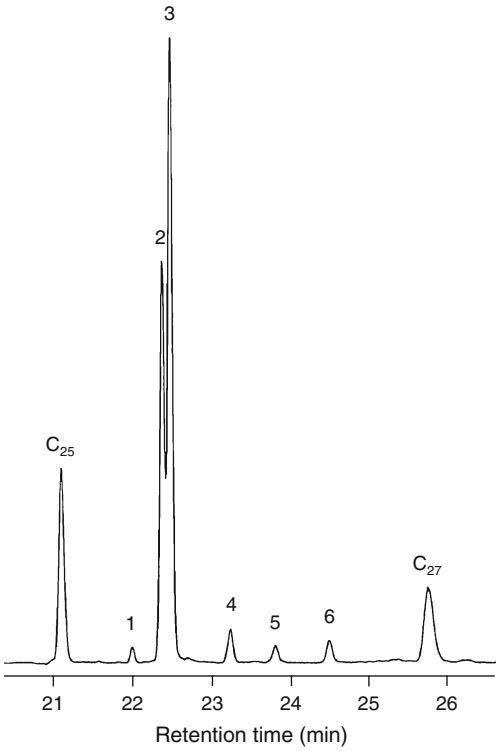


Fig. 1 Total ion chromatogram of GC-MS analysis of pheromone gland extract from female *Synanthedon bicingulata* on DB-Wax column. 1 (*Z*)-13-octadecenyl acetate, 2 (*E*,*Z*)-3,13-octadecadienyl acetate, 3 (*Z*,*Z*)-3,13-octadecadienyl acetate, 4 (*E*,*Z*)-2,13-octadecadienyl acetate, 5 (*E*,*Z*)-3,13-octadecadien-1-ol, 6 (*Z*,*Z*)-3,13-octadecadien-1-ol, C_{25} pentacosane, C_{27} heptacosane

indicating the original double bond at position 13 of the monounsaturated acetate (Buser et al., 1983). The calculated retention indices (RIs) of compound 1 coincided with *Z*13-18:OAc standard on two columns (KIs of 2548 on the DB-Wax column, and 2625 on the DB-23 column). The mass spectra of compounds 2 and 3 were similar and had diagnostic ions at *m/z* 308 (M^+), 248 (M-60), 219, 205, 191, and 61 suggestive of octadecadienyl acetates. The mass spectrum of the DMDS adduct of compounds in the pheromone extract showed a molecular ion *m/z* 496 (308+ 2MeSSMe) and diagnostic ions *m/z* 301, 255, 147, and 117, indicating the original double bonds at the 3- and 13-positions of octadecadienyl acetate (Vincenti et al. 1987). However, the geometry of the dienes was not confirmed by retention time comparisons because DMDS adducts of the isomers were poorly resolved on the DB-1 column. Comparison of RIs of the natural compounds with the 3,13 isomers of octadecadienyl acetates on the DB-Wax and DB-23 columns confirmed that compounds 2 and 3 were *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc, respectively (Table 1).

The mass spectrum of compound 4 showed diagnostic fragment ions of octadecadienyl acetate at *m/z* 248 (M-60), 219, and 61. Compounds 5 and 6 had a molecular ion *m/z* 266, and diagnostic fragment ions *m/z* 248 (M-18), 219, 205, and 191 suggestive of an octadecadien-1-ol. Whereas it was not possible to verify the structure of compounds 4, 5, and 6 using DMDS derivatization because of coelution with the large amount of unidentified products and/or the small amount of material in the derivatized gland extract, we were able to tentatively identify compounds 4, 5, and 6 as *E*2,*Z*13-18:OAc, *E*3,*Z*13-18:OH, and *Z*3,*Z*13-18:OH, respectively, by comparison of their RIs with those of synthetic standards on two columns. The relative ratio of compounds 1–6 in gland extracts was 2:58:100:6:3:4 (N=10).

Field Experiments In field experiment 1, a total of 181 male *S. bicingulata* were caught, with no catch in controls or traps baited with single components. The maximum number of males was attracted to a 4:6 mixture of *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc, although it was not significantly better than the 3:7 and 2:8 blends (Fig. 2). A total of 267 males were captured in field experiment 2. Trap catches of males to the binary blend of *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc were unaffected by the addition of the minor glandular components, *Z*13-18:OAc, *E*2,*Z*13-18:

Table 1 Retention indices of octadecadienyl compounds (compounds 2, 3, 4, 5 and 6) present in gland extracts of female *Synanthedon bicingulata* and synthetic 3,13- and 2,13-octadecadienyl compounds on DB-Wax and DB-23 columns

Compound	DB-Wax	DB-23
Female extract		
Compound 2	2569	2626
Compound 3	2574	2639
Compound 4	2610	2660
Compound 5	2633	2648
Compound 6	2660	2683
Authentic standard		
(*E*,*E*)-3,13-octadecadienyl acetate	2563	2606
(*E*,*Z*)-3,13-octadecadienyl acetate	2569	2626
(*Z*,*E*)-3,13-octadecadienyl acetate	2567	2618
(*Z*,*Z*)-3,13-octadecadienyl acetate	2574	2639
(*E*,*E*)-2,13-octadecadienyl acetate	2603	2636
(*E*,*Z*)-2,13-octadecadienyl acetate	2610	2660
(*Z*,*E*)-2,13-octadecadienyl acetate	2574	2618
(*Z*,*Z*)-2,13-octadecadienyl acetate	2581	2639
(*E*,*E*)-3,13-octadecadien-1-ol	2626	2627
(*E*,*Z*)-3,13-octadecadien-1-ol	2633	2648
(*Z*,*E*)-3,13-octadecadien-1-ol	2652	2661
(*Z*,*Z*)-3,13-octadecadien-1-ol	2660	2683
(*E*,*E*)-2,13-octadecadien-1-ol	2684	2674
(*E*,*Z*)-2,13-octadecadien-1-ol	2693	2698
(*Z*,*E*)-2,13-octadecadien-1-ol	2684	2693
(*Z*,*Z*)-2,13-octadecadien-1-ol	2693	2716

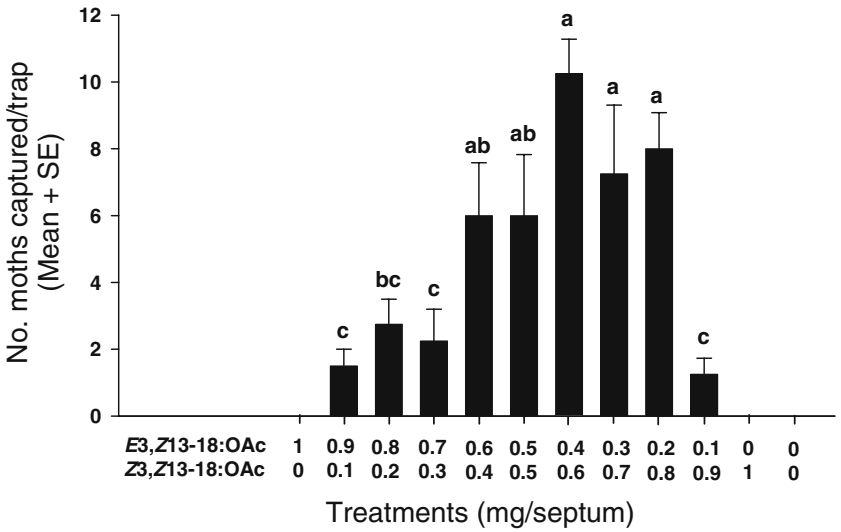


Fig. 2 Number of *Synanthedon bicingulata* males captured in traps baited with lures containing different ratios of *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc at peach orchards in Jeonju, Korea, 10–18 June 2009 (*N*=4). Bars with the same letter are not significantly different (Tukey's test: $P>0.05$)

OAc, *E*3,*Z*13-18:OH, and *Z*3,*Z*13-18:OH (Fig. 3). Moreover, the full six-component blend mimicking the blend found in a female gland extract did not increase trap catches compared with traps baited with the primary binary blend.

Discussion

Chemical analyses and field trials indicate that the sex pheromone of *S. bicingulata* consists of two major synergistic components, *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc, at a ratio of 4:6, respectively. In our previous study on the pheromone of the congeneric *S. haitangvora*, GC-MS analyses of the DMDS-derivatized extract conducted on a polar DB-Wax column did not show the presence of DMDS adducts (*m/z* 496) of octadecadienyl acetate derivatives (Yang et al., 2009b). The failure of the previous attempt probably was because the tetrasubstituted linear high-boiling derivatives are not eluted on the polar column (Vincenti et al., 1987). In this study, we found that DMDS adducts of octadecadienyl acetates eluted on the nonpolar DB-1 column. Thus, DMDS adducts can be useful in pheromone identification of sesiid species.

*E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc have been identified as constituents of a sex pheromone in several other sesiid species (El-Sayed, 2010). However, *Synanthedon hector* is the only previously reported sesiid species that uses a mixture of these two dienes as its sex pheromone. *Synanthedon hector* is distributed only in Japan, and is an important economic pest of cultivated *Prunus* spp. This species can be distinguished easily from *S. bicingulata* based upon the color patterns of the adult abdominal

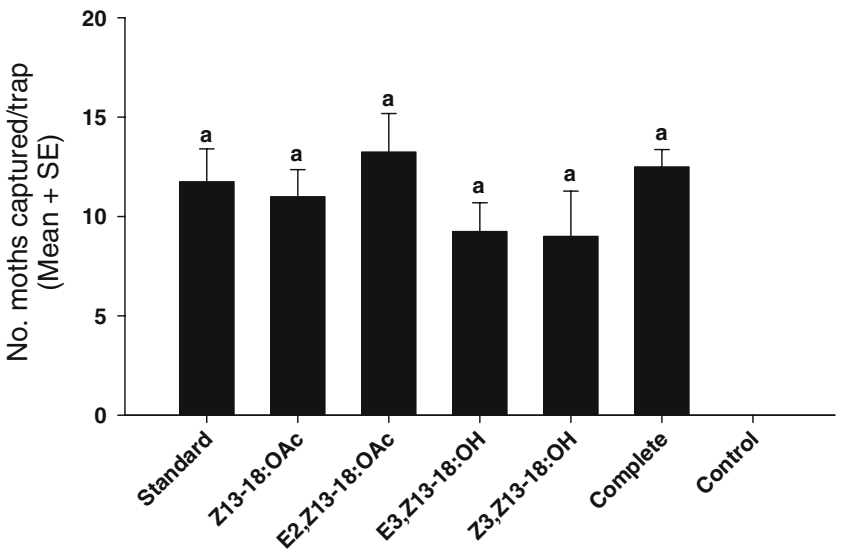


Fig. 3 The effect of adding 3% of different minor components to standard baits with 1 mg/septum of a 4:6 blend of *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc on captures of male *Synanthedon bicingulata* at peach orchards in Jeonju, Korea, 19–28 June 2009 (*N*=4). The complete blend consisted of all six components in a ratio found in the pheromone gland extract. Bars with the same letter are not significantly different (Tukey's test: $P>0.05$)

sternites (Arita et al., 2004). The pheromone glands of female *S. hector* contain *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc at 38:62 ratio (Naka et al., 2008), and conspecific males are optimally attracted to 5:5 blend (Yaginuma et al., 1976; Naka et al., 2008). These data show that allopatric *S. bicingulata* and *S. hector* utilize the same sex pheromone and host plants.

*Z*13-18:OAc, *E*2,*Z*13-18:OAc, *E*3,*Z*13-18:OH, and *Z*3,*Z*13-18:OH were identified as minor components of female *S. bicingulata* gland extracts, but addition of these components to the two major components had no significant effect on attraction of conspecific males. These minor components may serve an antagonistic function to suppress attraction of sympatric species. However, it is not known whether they are actually released from the female glands during calling. Previous studies with other sesiid species have shown that geometrical isomers or biosynthetic precursors of the major components are present in female pheromone glands, but the biological significance of these components remain unclear (Klun et al., 1990; Francke et al., 2004; Mozûraitis et al., 2006; Yang et al., 2009b). Hence, *Z*13-18:OAc and *E*2,*Z*13-18:OAc may just be by-products of the pheromone biosynthetic pathway, and *E*3,*Z*13-18:OH and *Z*3,*Z*13-18:OH are presumed to be biosynthetic precursors of the two major components, *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc, respectively.

During the course of this study, we found that no other sesiid species were attracted to a 4:6 mixture of *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc. In Korea, the genus *Synanthedon* is composed of 12 species (Arita et al. 2004), but sex pheromones or attractants have been reported for only three species, *S. haitangvora, S. tenuis* and *S. quercus* (Tamaki et al. 1977; Yang et al. 2009b). As shown in Fig. 2, *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc were essential for attraction of male *S. bicingulata*. This result suggests that *S. bicingulata* males may not be attracted to *S. haitangvora* females that emit *Z*3,*Z*13-18:OAc and *E*2,*Z*13-18:OAc as its pheromone (Yang et al., 2009b). In field screening trials for *S. tenuis*, Tamaki et al. (1977) found an antagonistic effect of *E*3,*Z*13-18:OAc on male attraction when it was mixed with the major component, *Z*3,*Z*13-18:OAc, in a very low ratio. Therefore, *E*3,*Z*13-18:OAc may prevent cross-attraction between *S. bicingulata* and *S. tenuis* where they occur sympatrically. They also confirmed that *E*3,*Z*13-18:OAc and *Z*3,*Z*13-18:OAc attracted few *S. quercus* males when tested separately or in binary mixture. We, therefore, suggest that specific chemical signals contribute strongly to the maintenance of premating reproductive isolation between sympatric populations of *Synanthedon* species.

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Variable Responses by Southern Pine Beetle, *Dendroctonus frontalis* Zimmermann, to the Pheromone Component *endo*-Brevicomin: Influence of Enantiomeric Composition, Release Rate, and Proximity to Infestations

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Abstract The male-produced bicyclic acetal *endo*-brevicomin is a component of the pheromone blend that mediates colonization of host pines by the bark beetle *Dendroctonus frontalis* Zimmermann. Efforts to identify its behavioral function have been complicated by contrasting reports that it either enhances or reduces attraction of flying beetles. Our studies failed to support the hypothesis that this published variability is due to differences in release rate and/or the enantiomeric composition [i.e., the beetle-produced (+)-enantiomer vs. the racemate] of the *endo*-brevicomin used in the experiments. In trapping trials within active *D. frontalis* infestations, racemic and (+)-*endo*-brevicomin did not differ from each other in behavioral effects when tested at seven different release rates ranging from 0.005 to 3 mg/d. At the highest release rates, racemic and (+)-*endo*-brevicomin similarly reduced catches in traps baited with an attractant (frontalin and turpentine), but neither enhanced catches at any release rate. Furthermore, the activity of racemic *endo*-brevicomin baits depended on trap proximity to *D. frontalis* infestations. Addition of these baits to attractant-baited traps located inside active infestations reduced catches, but they enhanced catches at traps located either 100 or 200 m outside these infestations. The contrasting responses may reflect differences in host-seeking strategies by either aggregated or dispersing *D. frontalis,* and may be elicited by differing abundance of natural sources of semiochemicals or differing responsiveness of beetles inside vs. outside of infestations. We suspect that much of the published variability in *D. frontalis* responses to *endo*-brevicomin is attributable to differing proximity of experimental field sites to infestations.

Key Words Aggregation · Attractant antagonist · Coleoptera · Dispersal · Enantiomer · Flight behavior · Pheromone synergist · Scolytidae · Semiochemical · Spatial dynamics · Trapping

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Introduction

Aggressive species of bark beetles (Coleoptera: Scolytidae) overcome host defenses by attacking trees in large numbers, and thus are able to colonize trees that would not be susceptible to infestation by smaller numbers of insects (Paine et al., 1997). These aggressive species stimulate and synchronize such "mass attacks," which inevitably result in death of the host tree, by releasing pheromones whose activity is often modified by compounds from the tree itself (Wood, 1982; Byers, 1989). Pheromones of aggressive bark beetles likely fulfill at least two functions in the mass-colonization process: as aggregation pheromones that attract beetles from the general area and concentrate landings and attacks on one or a few trees, and as

antiaggregation pheromones that terminate aggregation on a fully-colonized host and prevent overcrowding (Borden, 1982). Due to the essential role that these semiochemicals play in the beetles' ability to kill trees, substantial effort has been invested in developing semiochemical-based management techniques (Borden, 1995).

The southern pine beetle, *Dendroctonus frontalis* Zimmermann, is an aggressive bark beetle pest of pines in the southeastern United States, Mexico, and much of Central America (Payne, 1980). Population outbreaks are conspicuous as spatially discrete infestations or "spots," which are zones of contiguous, currently-infested or dead, abandoned trees surrounded by healthy forest (Franklin, 1970; Thatcher and Barry, 1982; Clarke and Nowak, 2010). During summer, brief (i.e., ~40 d), broadly-overlapping generations of brood beetles emerge from previously-infested trees within these infestations and are attracted to semiochemicals released from newly-infested trees at the leading edge of the growing infestation (Gara et al., 1965; Gara and Coster, 1968; Billings and Pase, 1979). These beetles either attack non-colonized areas of the main stem on these newly-infested trees or initiate colonization of adjacent, uninfested trees. Infestations accumulate new trees typically along just one side, and thus normally have a single, well-defined "head" and trajectory of infestation growth (i.e., the direction of newly-infested trees relative to those previously infested) at any given time (Coster et al., 1978; Schowalter et al., 1981).

The bicyclic acetal *endo*-brevicomin appears to be a key component of the semiochemical blend that mediates *D. frontalis* aggregation and infestation growth. Male beetles pairing with attack-initiating females in the host bark release apparently enantiomerically pure (+)-*endo*-brevicomin (Sullivan et al., 2007). Baits of synthetic (+)-*endo*-brevicomin can greatly enhance responses by both sexes to traps baited with pine volatiles and the female-produced attractant frontalin (Vité et al., 1985; Sullivan et al., 2007), implying that (+)-*endo*-brevicomin is a component of the aggregation pheromone. Furthermore, *endo*-brevicomin baits can have an even greater catch-enhancing effect when displaced 4–16 m away from such traps (Sullivan and Mori, 2009). Thus, in addition to enhancing attraction to the tree of origin, male-produced *endo*-brevicomin may mediate switching of the focus of mass attack from fully colonized trees (i.e., ones with many established pairs) to adjacent trees receiving initial attacks by females (Sullivan and Mori, 2009).

This interpretation of the biological function of *endo*-brevicomin is based upon results of behavioral studies performed with the beetle-produced (+)-enantiomer (Vité et al., 1985; Sullivan et al., 2007; Sullivan and Mori, 2009). However, the potent attractant synergism observed for (+)-*endo*-brevicomin in published studies contrasts sharply with several reports that racemic *endo*-brevicomin baits can inhibit *D. frontalis* responses to traps baited with frontalin and host odors (Vité and Renwick, 1971; Payne et al., 1978a; Salom et al., 1992). The inhibitory activity of racemic *endo*-brevicomin was reported earlier than the synergistic capacity of (+)-*endo*-brevicomin, and this led to the original classification of *endo*-brevicomin as a component of an antiaggregation pheromone for *D. frontalis* (Borden, 1982, 1985; Skillen et al., 1997). Vité et al. (1985) hypothesized that the reported inhibitory activity of *endo*-brevicomin was due to earlier studies' use of high release rates of the racemate and the presence of the (−)-enantiomer, which they demonstrated could inhibit *D. frontalis* responses to its attractant. However, no single, controlled study has evaluated this hypothesis by directly comparing *D. frontalis* responses to racemic and (+)-*endo*-brevicomin across a range of doses.

Furthermore, there are other possible reasons for the disparity among published results of field studies on *D. frontalis* responses to *endo*-brevicomin. Those trapping studies that demonstrated potent attractant synergism for *endo*-brevicomin generally were performed in uninfested tracts of forest (i.e., Sullivan et al., 2007; Sullivan and Mori, 2009; half of replicates in Vité et al., 1985); whereas those that demonstrated inhibition were performed mostly inside of growing infestations (i.e., Payne et al., 1978a; Salom et al., 1992; not detailed in Vité and Renwick, 1971). Host seeking behavior of *D. frontalis* ostensibly differs inside and outside of active infestations (Gara, 1967; Payne and Coulson, 1985); hence flying *D. frontalis* may respond differently to sources of *endo*-brevicomin located within or close to infestations rather than some distance away.

In this study, we tested the hypothesis that variation in the reported behavioral activity of *endo*-brevicomin can be attributed simply to variation in the enantiomeric composition and/or dose of the baits by comparing the flight response of *D. frontalis* to a range of release rates of racemic and (+)-*endo*-brevicomin in the field. Secondly, we tested the hypothesis that proximity of an active infestation affects the flight response of *D. frontalis* to *endo*-brevicomin by simultaneously operating attractant-baited traps inside and outside of infestations and altering the presence of an *endo*-brevicomin bait.

Methods and Materials

All experiments utilized multiple-funnel traps (12-unit; ChemTica Internacional, S.A., San Jose, Costa Rica) that were suspended from vertical standards consisting of 1.7 cm diam pieces of electrical conduit staked into the ground. Each trap cup was positioned 1–1.5 m above the ground and contained several centimeters of propylene glycol and water (1:3) to retain and preserve captured insects.

Experiment 1: Variable Dose and Enantiomeric Composition of endo-Brevicomin Baits Deployed Inside of Infestations Sixteen different trap bait treatments were compared: an unbaited trap, a standard attractant consisting of frontalin and pine turpentine, or the standard attractant plus one of seven logarithmically increasing release rates of either (+)- or racemic *endo*-brevicomin (Table 1). *endo*-Brevicomin was released either from glass capillaries with one heat-sealed end, or from open, 100 μl-capacity glass autosampler vial inserts (Table 1). Capillaries and inserts were secured open-end-up inside of an uncapped, inverted 4 ml-capacity screw-cap glass vial (Sullivan and Mori, 2009). One device was deployed per trap for the (+)-*endo*-brevicomin treatments; two devices were deployed for the racemic treatments. Hence, the release of (+) was equal for both enantiomeric treatments at each level of release rate. (+)-*endo*-Brevicomin was synthesized (Sullivan et al., 2007) and was >99% pure (enantiomerically and chemically) by NMR spectroscopy and GC. There were no detected contaminants with known behavioral activity. Racemic *endo*-brevicomin [Phero Tech International (now Contech Enterprises Inc.), Delta, BC, Canada] was 95% chemically pure by GC (<1% *exo*-brevicomin contamination). Racemic frontalin was released from a pair of capped 400 μl-capacity LDPE microcentrifuge tubes each containing 200–300 μl frontalin [>95% chemical purity (contaminants with no known behavioral activity), ChemTica]. Turpentine (steam distilled from loblolly pine, *Pinus taeda* L.; Hercules Inc., Brunswick, GA, USA; composition determined by GC-FID as 72% α-pinene, 1% *trans*-verbenol, 1% *trans*-pinocarveol, <1% myrtenal, myrtenol, and verbenone, with the remainder consisting of 5% β-pinene, 6% camphene, and other monoterpenes of no known behavioral activity) was released from a pair of LDPE transfer pipettes filled to capacity (4 ml) and heat-sealed at the tip. Bait release rates were measured in a fume hood at 24±2°C (23±2°C for frontalin), either gravimetrically for turpentine (0.12 g/d) and frontalin (5 mg/d) or by volume loss for *endo*-brevicomin (Table 1). Baits were attached at the fourth funnel from the bottom of the trap.

Sixteen traps were placed inside each of two actively-growing *D. frontalis* infestations (located >1 km apart) in stands of *P. taeda*, in the Homochitto National Forest, Mississippi. Traps were clustered (i.e., with no particular geometric arrangement) within portions of the infestations occupied by trees with predominantly larval-stage *D. frontalis* brood (Payne et al., 1978b), and were <10 m away from a brood tree and <50 m away from trees undergoing mass attack and presumably releasing aggregation semiochemicals. Traps were spaced >1 m from the nearest pine and 5–10 m apart; this close trap spacing was required in order to test all 16 treatments simultaneously within each infestation. The 16 bait treatments were assigned randomly and without duplication to the traps of each infestation. Trap catch was collected after 1–4 d, and then the treatment assignments were re-randomized without replacement to any previous position. Randomization and catch collection were repeated 9 times at one of the infestations (5–19 July 2005) and 7 at the other (5–13 July 2005; we were required to abandon this latter infestation so it could be controlled).

Mean daily catch for both males and females was calculated for every collection. All trap catch data for both experiments 1 and 2 were cube root transformed to meet the distributional assumptions of the tests; conformity to assumptions was confirmed by examination of residuals plots. Catches by the unbaited traps were omitted from the statistical analyses (Reeve and Strom, 2004). A mixed model ANOVA was carried out by using PROC MIXED (SAS 9.0, SAS Institute Inc., Cary, NC, USA) with effects for treatment, sex, and sex by treatment assumed fixed. Infestation and date within infestation were considered random sources of replication, and so treatment by infestation and treatment by date within infestation were also random. Sex was viewed as a repeated measures factor within each trap and date combination, hence sex by treatment by infestation, and sex by date within infestation, were also included as random effects. Treatment was partitioned to test for a significant effect of release rate, enantiomeric composition of *endo*-brevicomin, and interactions between these factors. Treatment by sex was partitioned analogously. An ESTIMATE statement was used to test for an interaction between sex and presence/absence of *endo*-brevicomin (averaged for both enantiomeric compositions). Degrees of freedom were obtained by using the default containment option. Since no significant interactions between treatment and sex were found, total catches of *D. frontalis* (i.e., with males and females pooled) were cube root transformed and subjected to the same mixed model ANOVA with omission of all effects involving sex. ESTIMATE statements were used to compare mean catches (averaged over enantiomeric compositions) at each release rate to catches by the standard attractant alone, with a Bonferroni correction applied to the resulting *P*-values (α=0.05).

Experiment 2: endo-Brevicomin Activity Inside vs. Outside of Infestations Tests were performed in 16 *D. frontalis* infestations; 8 in the Oconee National Forest, Georgia (25 June–26 July 2007) and 8 in the Homochitto National Forest, Mississippi (9 August–21 September 2007). Selected infestations had at least 5 recently-infested trees (i.e., with green crowns) and at least one tree visibly undergoing mass attack at the infestation head when trapping was initiated. All but one of the infestations continued to accumulate new, infested trees during the trapping period, but in all cases, the infestation heads advanced less than 20 m. Three traps were placed at each infestation: (1) inside the infestation head (surrounded by trees containing egg-stage brood and 4–20 m

Table 1 Construction and elution rate of *endo*-brevicomin release devices for experiment 1

Dose level	Glass insert/capillary characteristics				Elution rate[a] (mg/d ± s.d.)
	Diameter (i.d. mm)	Length (mm)	Height filled (mm)	Number per device	
1)	0.200	32	10	1	0.0049±0.0001
2)	0.346	32	10	1	0.015±0.001
3)	0.631	32	10	1	0.049±0.001
4)	1.17	32	10	1	0.16±0.01
5)	1.17	32	10	3	0.47±0.02
6)	3.66	30	10	1	1.2±0.1
7)	3.66	30	8	3	3.0±0.2

Each device consisted of 1–3 glass autosampler vial inserts or glass capillaries secured open-end-up inside of an inverted, uncapped 4 ml capacity screw-cap vial

[a] Measured with the racemate by volume loss in a fume hood over 26 d at 24±2°C (N=3). One device was deployed per trap for the (+)-*endo*-brevicomin treatments; two devices were deployed per trap for the racemic *endo*-brevicomin treatments. Hence, this rate represents the release of each enantiomer of *endo*-brevicomin

from trees being mass attacked); 2) 100 m; and 3) 200 m beyond the limit of infested trees and within forests with a pine component within the general trajectory of infestation growth. In some instances, trap locations diverged from the trajectory of infestation growth as much as 90° if land within the infestation trajectory did not contain susceptible pines or was not publicly owned. Due to the inherently limited size of forest management units ("stands"), it was often necessary to locate the 2nd or 3rd (i.e., the 100 or 200 m) traps within adjacent forest stands that may not have been identical in tree composition, age, density, and other characteristics to the stand containing the beetle infestation itself. In some cases, the stand containing the infestation and those containing the 2nd or 3 rd traps were separated by treeless zones such as roads and utility right-of-ways. No multiple-tree infestations were closer to the 2nd and 3rd traps than the selected infestation, and no solitary colonized trees were closer than 50 m. Traps typically were placed no closer than 4 m from healthy pines to reduce chances of artificially inducing new infestations in these trees, but distances as small as 2 m were unavoidable in high-density pine stands.

All three traps at an infestation were consistently baited with frontalin and turpentine. A device releasing racemic *endo*-brevicomin was either attached simultaneously to all 3 traps assigned to an infestation or not attached to any, with the initial treatment (*endo*-brevicomin present/absent) chosen by a coin toss. Catch was collected at intervals of 1–5 d, and the treatment assignment was reversed for all 3 traps at an infestation on each date that catch was collected (3–10 collections over a 5–26 d trapping period). The frontalin bait was the same as experiment 1 except a single tube was used (i.e., 2.5 mg/d at 23°C). The turpentine bait (4–7 g/d at 24°C) was a single 250 ml-capacity brown glass bottle with a 1 cm diam piece of cotton dental wick immersed in the liquid (200–250 ml, same source and chemical composition as in experiment 1) and extending 1–2 cm through a hole in the cap. The *endo*-brevicomin bait (0.5–0.8 mg/d at 24°C) was an LDPE "bubble"-type device (ChemTica) containing 49 mg racemic *endo*-brevicomin (>95% purity with 2–3% *exo*-brevicomin). Frontalin and *endo*-brevicomin baits were attached near the center of each trap, whereas the turpentine bottle was placed within the funnel immediately below the trap top to protect the wick from rain.

At each trap collection, pines adjacent to the 100 and 200 m traps were checked for the presence of pitch tubes and other evidence of *D. frontalis* attack. Small numbers of attacks (1–5) were sometimes observed on one or more nearby pines. However, if a mass attack were observed on one or more adjacent trees (as occurred at one infestation in Mississippi), catch data from this trap collection were discarded, and the trap was relocated >50 m away from the infested trees while maintaining the assigned treatment distance from the infestation.

For each trap, catches were pooled across collections to obtain a single mean value for daily catch with *endo*-brevicomin either absent or present. We performed a mixed-model ANOVA on these cube root transformed means by using PROC MIXED (SAS 9.0) with main and interaction effects for four fixed factors (*endo*-brevicomin treatment, trap position, state, and beetle sex) plus the following four random effects: infestation within state, trap position by infestation within state, *endo*-brevicomin treatment by infestation within state, and trap position by *endo*-brevicomin treatment by infestation within state. Thus, infestation within state was the random source of replication, whereas trap position and *endo*-brevicomin

treatment were considered stripped (i.e., applied uniformly) across each other within infestations. Sex was considered a repeated measures factor within each combination of trap position and *endo*-brevicomin treatment. To examine significant interactions, means for levels of one factor were compared at each fixed level of another factor by using the SLICE option with the LSMEANS statement. A Bonferroni correction was applied to the resulting *P*-values separately for each significant interaction (α=0.05).

Results

Experiment 1: Variable Dose and Enantiomeric Composition of endo-Brevicomin Baits Deployed Inside of Infestations No significant interactions were revealed between sex of the responding beetles and bait treatment overall (F=1.34; df=14,15; P=0.29), or between sex and the various treatment components, specifically, between sex and the presence/absence of *endo*-brevicomin (t=0.42; df=15; P=0.68); between sex and *endo*-brevicomin dose (F=1.87; df=7,15; P=0.15); between sex and the enantiomeric composition of the *endo*-brevicomin bait (F=0.00; df=1,15; P=0.97); or among sex, dose, and enantiomeric composition of *endo*-brevicomin (F=0.94; df=6,15; P=0.50). With both sexes of *D. frontalis* pooled and averaging over dose, the enantiomeric composition of the *endo*-brevicomin bait had no significant effect on trap catches (F=0.00; df=1,14; P=0.98), and catches were not influenced by an interaction between dose of *endo*-brevicomin and its enantiomeric composition (F=1.04; df=6,14; P=0.44). However, averaging over enantiomeric composition, the release rate of *endo*-brevicomin had a highly significant effect on trap catches (F=17.5; df=6,14; P<0.001). Traps with the two highest release rates of *endo*-brevicomin caught significantly fewer beetles than traps baited with the standard attractant alone (F≥14.8; df=1,14; Bonferroni adjusted P≤0.013; Fig. 1). Traps with lower release rates of *endo*-brevicomin did not catch beetles in numbers that differed significantly from the attractant-only traps (P>0.45).

Experiment 2: endo-Brevicomin Activity Inside vs. Outside of Infestations A highly significant two-way interaction was revealed between trap location relative to the infestation head and presence/absence of the *endo*-brevicomin bait (F=40.3; df=2,28; P<0.001). Averaging over states, *endo*-brevicomin significantly reduced *D. frontalis* catches at frontalin/turpentine baited traps located inside the head of the infestation, but significantly increased catches in such traps located 100 and 200 meters beyond the head of the infestation (Table 2). However, a marginally-significant three-way interaction was detected between presence/absence of *endo*-brevicomin, trap location relative to the infestation head, and the state where the infestations were located (F=2.97; df=2,28; P=0.068). With the two states considered separately, *endo*-brevicomin significantly reduced catches at traps within the infestation in both states, but it significantly enhanced catches at traps outside the infestation only at the 100 m distance in Georgia and the 200 m distance in Mississippi (Table 2). However, this interaction with state was a quantitative (i.e., non-crossover) interaction, and the mean trend in both states was catch enhancement by *endo*-brevicomin for both trap locations outside the infestations. There was no significant interaction between sex and the effect of *endo*-brevicomin: there was not a significant three-way interaction among sex, *endo*-brevicomin presence, and trap location relative to the infestation head (F=0.91; df=2,84; P=0.406) nor a significant four-way interaction among sex, *endo*-brevicomin presence, state, and trap location relative to the infestation head (F=1.92; df=2,84; P=0.153). However, there was a highly significant two-way interaction between trap location relative to the infestation head and sex (F=35.2; df=2,84; P<0.001), apparently due to more strongly male-skewed sex ratios trapped within the infestation than outside. Furthermore, there was as a highly significant interaction between state and sex (F=83.0; df=1,84; P<0.001), apparently from more strongly male-skewed sex ratios trapped in Georgia than in Mississippi.

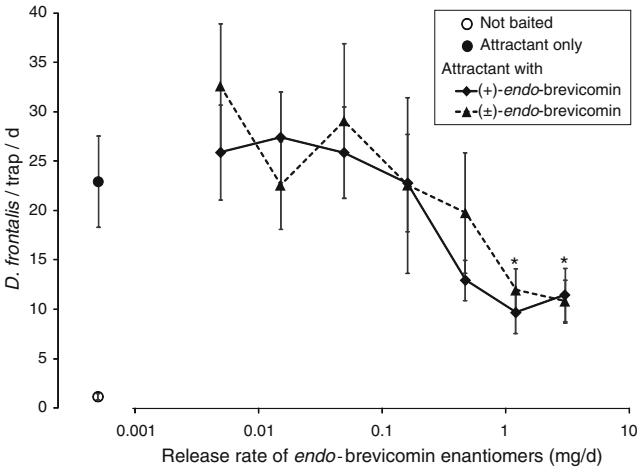


Fig. 1 Catches of *Dendroctonus frontalis* in multiple-funnel traps baited with an attractant (racemic frontalin and turpentine) alone or with varying release rates of either racemic or (+)-*endo*-brevicomin, Homochitto National Forest, Mississippi, July 2005. Catches at release rates of *endo*-brevicomin denoted with an asterisk were significantly different from catches by the attractant-only control trap (Proc Mixed ANOVA on transformed data; CONTRASTS with Bonferroni correction; α=0.05). The release rate indicated by the X-axis is for each individual enantiomer, thus the total release of *endo*-brevicomin [that is, the (+)- and (−)-enantiomers summed] by the racemic bait was twice that for the (+)-*endo*-brevicomin bait

Discussion

Experiment 2 demonstrated that an *endo*-brevicomin bait with a fixed release rate can produce apparently opposite behavioral effects depending on whether it is deployed inside or outside the head of an active *D. frontalis* infestation. The *endo*-brevicomin bait of experiment 2 consistently inhibited attraction and/or landing at point sources of attractant within infestations, but generally enhanced these responses outside. Furthermore, inside infestations we failed to observe attraction enhancement by either racemic or (+)-*endo*-brevicomin at any tested dose (experiment 1), including doses within a range that significantly enhanced catches in previous studies that we conducted outside of infestations [approximately 0.2–3 mg/d for (+); 0.5 mg/d for racemic; Sullivan et al., 2007; Sullivan and Mori, 2009].[1]

This variability may in part explain the alternatively synergistic or inhibitory activity reported by the six published trapping studies with *D. frontalis* that investigated the attractant-modifying capacity of *endo*-brevicomin alone. Although there was variation in the enantiomeric composition and release rate of the deployed baits, two of these studies performed away from infestations reported attraction enhancement or no effect by *endo*-brevicomin (Sullivan et al., 2007; Sullivan and Mori, 2009), whereas two performed inside infestations reported either attractant inhibition or no effect (Payne et al., 1978a; Salom et al., 1992). Vité and Renwick (1971) observed attractant inhibition, but did not report proximity of their traps to infestations. However, our results appear inconsistent with those of Vité et al. (1985), who observed significant attraction enhancement by both racemic and (+)-*endo*-brevicomin with half of their replicates conducted "within (a *D. frontalis*) infestation" and half in "uninfested stands." Although they pooled data from these two locations for their statistical analyses, their means reported for each site suggest similar responses to *endo*-brevicomin.

Furthermore, modest inconsistencies in our data from experiment 2 suggest that caution be used in inferring that *endo*-brevicomin invariably has opposite activities inside vs. outside of *D. frontalis* infestations. Significant catch enhancement by *endo*-brevicomin at both distances outside of infestations (100 m and 200 m) was detected only when data from both Mississippi and Georgia were pooled, and between the two states there appeared to be no consistent relationship between distance from the infestation and the degree of the enhancement effect of *endo*-brevicomin. The

[1] It should be noted that experiment 1 and the tests cited in Sullivan et al. (2007) and Sullivan and Mori (2009) differed substantially in the rate of turpentine released from the standard bait (i.e., 0.12 vs. 7 g/d, respectively).

Table 2 Effect of *endo*-brevicomin baits on *Dendroctonus frontalis* flight responses to attractant-baited traps positioned either inside or outside of active infestations

Trap location	*endo*-Brevicomin bait	Homochitto Nat. For., Mississippi Aug.–Sept., 2007 (N=8)[a]			Oconee Nat. For., Georgia June–July, 2007 (N=8)[a]			States combined (N=16)[a]		
		Catch/trap/d	Proportion females	P[b]	Catch/trap/d	Proportion females	P[b]	Catch/trap/d	Proportion females	P[b]
Inside infestation	Present	38.5±14.9	0.35	0.010	67.3±29.8	0.12	<0.001	52.9±16.5	0.23	<0.001
	Absent	114.9±63.1	0.43		221.0±108.8	0.16		168.0±62.3	0.30	
100 m outside	Present	9.7±4.1	0.47	0.197	97.8±47.1	0.29	0.005	53.8±25.5	0.38	0.001
	Absent	3.9±2.5	0.45		26.3±10.8	0.30		15.1±6.1	0.37	
200 m outside	Present	26.3±7.7	0.51	<0.001	34.5±16.3	0.26	0.370	30.4±8.8	0.39	<0.001
	Absent	1.6±0.4	0.53		17.3±10.8	0.50		9.5±5.6	0.51	

Table entries represent untransformed trap catches (mean ± s.e.)

Attractant consisted of racemic frontalin and steam distilled *Pinus taeda* turpentine (see Methods and Materials for details)

[a] Each replicate was a single infestation of *D. frontalis*

[b] P-value for test of hypothesis that catch was the same with *endo*-brevicomin either present or absent at the trap (SLICE option of the LSMEANS statement, SAS PROC MIXED on cube root transformed data, with Bonferroni correction, α=0.05)

marginally-significant interaction detected between treatment effect and state may be related to large differences in the abundance of infestations in the forests utilized in either state: during the Summer of 2007 approximately 1.6 infestations/km^2 were detected on the Oconee National Forest, Georgia, but only 0.12/km^2 in the Homochitto National Forest, Mississippi (Valli Peacher, USFS-FHP, personal communication).

The contrasting activities of *endo*-brevicomin possibly reflect the very different demands on host/mate-seeking *D. frontalis* either inside or outside of infestations. Beetles dispersing outside infestations may be hundreds of meters from the nearest suitable host (Coulson et al., 1985), and natural selection should presumably favor strategies that reduce energy and time consumed during host location. For these insects, the combination of pheromone components of both sexes (i.e., frontalin and *endo*-brevicomin) with host odors may serve as a more reliable indicator of an established infestation—where high densities of attacking conspecifics assures continuous host and mate availability—than frontalin and host odors alone (Sullivan and Mori, 2009). For beetles flying within infestations, by contrast, host trees are immediately available through the continuous cycle of new mass attacks on adjacent, healthy trees at the infestation head (Thatcher, 1960; Gara et al., 1965). In this circumstance, individual reproductive success may be maximized through discrimination of phloem resource availability and quality within individual hosts undergoing mass attack. *endo*-Brevicomin may aid beetles in avoiding trees that have reached their colonization capacity (i.e., ones having many established pairs and presumably releasing elevated quantities of *endo*-brevicomin) in favor of incompletely colonized trees (i.e., ones with at least some solitary females releasing frontalin and fewer or no *endo*-brevicomin producing males). Thus, *endo*-brevicomin may have distinct functions for dispersed and aggregated *D. frontalis*, aiding the former in locating active infestations and the latter in assessing resource availability within individual hosts.

A range of variables relating to the environment, the physiological state of responding insects, and the chemical signal itself can ultimately determine how bark beetles respond to semiochemicals (Borden et al., 1986; Raffa, 2001; Miller et al., 2005; Pureswaran et al., 2008). Pheromone components that alternately exhibit either attractive or inhibitory activity within a single population have been identified in several bark beetle species (Rudinsky, 1973; Borden et al., 1987; Schlyter et al., 1987; Seybold et al., 1992). In these reports, the precise activity of such "multifunctional" pheromone components is governed by a quality intrinsic to the pheromone source, namely the release rate of the compound, with low release rates being attractive but high ones inhibitory (Borden, 1996). In our experiment 2, however, the experimentally deployed *endo*-brevicomin baits were identical both inside and outside of the *D. frontalis* infestations, hence the reversal of activity we observed in either situation ostensibly had causes extrinsic to the baits themselves. These might include:

(a) *Differing background semiochemicals associated with the traps.* A prevailing background of *D. frontalis* semiochemicals presumably exists within the microenvironment of infestations but not in the surrounding forest. Trees within the advancing head of an infestation may contain many thousands of pheromone-producing beetles (Pureswaran et al., 2008), and evidence indicates that the associated semiochemical plume can profoundly influence behavior of *D. frontalis* orienting within and near these infestations (Gara et al., 1965; Gara, 1967; Cronin et al., 1999). These background semiochemicals conceivably could alter beetle responses to point sources of semiochemicals established within the infestation, such as our baits. This possibility is suggested by the observation that a device releasing approximately a single infested tree equivalent of *endo*-brevicomin can alter *D. frontalis* catches in traps within a radius of at least 30 m (Sullivan and Mori, 2009), a distance that is greater than what separated the infestation-imbedded traps of our experiments and the nearest mass-attacked trees. It should be noted that in experiment 1 the presence of *endo*-brevicomin and the other bait components on adjacent treatments also may have influenced trap catches, since intertrap spacing was merely 5–10 m. However, we presume that the 100 m spacing among traps in experiment 2 was sufficient to preclude any significant cross-interference by experimental baits.

(b) *Relative abundance of competing sources of beetle attraction.* Nearby, alternative sources of attractive cues and landing sites (namely, mass-attacked trees) are readily available to beetles inside infestations but not outside. Thus, whether or not an orienting beetle lands in a trap within an infestation is likely influenced by the bait's attractiveness relative to these competing, attractive sources. One effect of *endo*-brevicomin is apparently to render collocated sources of attractant relatively less attractive than adjacent sources of attractant: when one of a group of adjacent frontalin/turpentine-baited traps is baited additionally with *endo*-brevicomin, this trap becomes relatively less attractive to *D. frontalis* than the adjacent traps lacking *endo*-brevicomin, even though catch is enhanced in all traps (Sullivan and Mori, 2009). Thus, in our experiments, the absence/presence of an *endo*-brevicomin bait on traps inside the infestations could have rendered the traps more/less attractive than nearby infested trees, altering the

number of beetles trapped. By contrast, there were no adjacent mass-attacked trees to serve as alternative, attractive targets to the baited traps located outside infestations in experiment 2.

(c) *Differing responsiveness of dispersed* vs. *aggregated individuals*. Evidence suggests that flying beetles within *D. frontalis* infestations are predominantly newly-emerged brood or re-emerged parent adults from trees within the infestation (Gara, 1967; Cronin et al., 1999). Thus, beetles responding to traps located within infestations likely had flown a shorter average distance and spent less time outside of a host than beetles responding to traps located outside the infestations. Flight exercise alters *D. frontalis* responses to aggregation semiochemicals (Andryszak et al., 1982), and both flight exercise and changes in fat reserves have been shown to alter semiochemical responses in other bark beetle species (Borden et al., 1986). Furthermore, one study found that *D. frontalis* trapped outside of infestations express genes in different frequencies than those trapped inside (Florence et al., 1982), suggesting that dispersing and aggregated beetles differ in their inherent physiological and, perhaps, behavioral capacities. However, this finding is cast into some doubt by the study's failure to examine the sexes separately and by our evidence from experiment 2 that sex ratios trapped inside and outside infestations differ substantially.

Vité et al. (1985) reported that pure (+)-*endo*-brevicomin baits enhanced, whereas pure (−)-*endo*-brevicomin baits reduced, *D. frontalis* responses to traps baited with frontalin and host odors. However, in infested stands (experiment 1), we failed to observe a significant difference in beetle response to either racemic or (+)-*endo*-brevicomin at any of seven release rates spanning nearly four orders of magnitude. Likewise, in a previous study performed in uninfested stands, racemic and (+)-*endo*-brevicomin released at approximately 0.23 mg/d did not differ in their capacity to enhance attraction of *D. frontalis* (Sullivan and Mori, 2009). Thus, despite evidence that (−)-*endo*-brevicomin can inhibit attraction by *D. frontalis* (Vité et al., 1985), we did not find that it modified the activity of the (+)-enantiomer in racemic baits. For this reason, we chose to use the more readily-available and less expensive racemic *endo*-brevicomin rather than (+)-*endo*-brevicomin in experiment 2.

We believe this is the first report of a bark beetle semiochemical bait reversing its apparent behavioral effect (i.e., between attraction enhancement and inhibition) due to its proximity to sites of beetle aggregation. This particular expression of multifunctionality in bark beetle semiochemicals could obviously complicate efforts to use them in beetle management, since failure to deploy such compounds in the appropriate context (i.e., either within infestations or within uninfested stands) could result in the opposite of the intended effect. Efforts to develop *endo*-brevicomin as a management semiochemical for *D. frontalis* have been frustrated by unintended effects, but ones apparently unrelated to *endo*-brevicomin's multifunctionality. Blends of *exo*- and *endo*-brevicomin can greatly reduce *D. frontalis* attacks on trees within infestations (Payne et al., 1977), suggesting these compounds might have value as tree protectants. However, trees within infestations treated with these two isomers of brevicomin experience reciprocal increases in attacks by secondary *Ips* spp. DeGeer bark beetles and therefore no reduction in mortality (Payne and Richerson, 1979; Richerson and Payne, 1979; Watterson et al., 1982). Although, tree protection with *endo*-brevicomin has not been attempted outside of infestations, our results lead us to expect that such treatment would also fail because it would likely enhance, rather than reduce, risk of *D. frontalis* attack.

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Identification of Components of the Female Sex Pheromone of the Simao Pine Caterpillar Moth, *Dendrolimus kikuchii* Matsumura

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Abstract The pine caterpillar moth, *Dendrolimus kikuchii* Matsumura (Lepidoptera: Lasiocampidae), is a pest of economic importance on pine in southwest China. Three active compounds were detected during analyses of solvent extracts and effluvia sampled by solid phase microextraction (SPME) from virgin female *D. kikuchii* using gas chromatography (GC) coupled with electroantennographic (EAG) recording with antennae from a male moth. The compounds were identified as (5*Z,*7*E*)-5,7-dodecadien-1-yl acetate (*Z*5,*E*7-12:OAc), (5*Z,*7*E*)-5,7-dodecadien-1-ol (*Z*5,*E*7-12:OH), and (5*Z*)-5-dodecenyl acetate (*Z*5-12:OAc) by comparison of their GC retention indices, mass spectra, and EAG activities with those of synthetic standards. Microchemical reactions of gland extracts provided further information confirming the identifications of the three components. Solvent extractions and SPME samples of pheromone effluvia from virgin calling females provided 100:18:0.6 and 100:7:1 ratios of *Z*5,*E*7-12:OAc:*Z*5,*E*7-12:OH:*Z*5-12:OAc, respectively. Field behavioral assays showed that *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH were essential for attraction of male *D. kikuchii* moths. However, the most attractive blend contained these three components in a 100:20:25 ratio in a gray rubber septa. Our results demonstrated that the blend of *Z*5,*E*7-12:OAc, *Z*5,*E*7-12:OH, and *Z*5-12:OAc comprise the sex pheromone of *D. kikuchii*. The optimized three-component lure blend is recommended for monitoring *D. kikuchii* infestations.

Key Words *Dendrolimus kikuchii* · (5*Z,*7*E*)-5,7-dodecadien-1-yl acetate · (5*Z,*7*E*)-5,7-dodecadien-1-ol · (5*Z*)-5-dodecenyl acetate · Field behavioral assay · Lasiocampidae · Lepidoptera · Sex pheromone · Solid phase microextraction

Introduction

Pine caterpillars (Lepidoptera: Lasiocampidae) are a serious pest of pine in China. Until now, about eighty species from seven genera have been identified (Hou, 1987). Species in the genus *Dendrolimus* Germar, including the six principal species *Dendrolimus punctatus* (Walker), *D. tabulaeformis* Tsai et Liu, *D. spectabilis* (Butler), *D. superans* Butler, *D. houi* Lajonquière, and *D. kikuchii* Matsumura, are considered to be most harmful, with populations that frequently reach outbreak status and cause significant damage to forests throughout China (Hou, 1987). The Simao pine caterpillar moth, *D. kikuchii*, is broadly distributed across southern China and causes severe damage to *Pinus yunnanensis* Franch., *P. kesiya* var. *langbianensis* (A. Chev.), *P. massoniana* Lamb., *P. armandii* Franch., *P. thunbergii* Parl., *P. taiwanensis* Hayata, *Keteleeria evelyniana* Mast., and other trees in Anhui, Jiangxi, Hunan, Hubei, Fujian,

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Zhejiang, Jiangsu, Yunnan, and portions of several other Chinese provinces (Xiao, 1991). The number of generations per year of *D. kikuchii* varies geographically. In Simao prefecture in southern Yunnan province, *D. kikuchii* has two generations per year, but in the northern part of Yunnan province only one generation occurs (Xiao, 1991). In Simao prefecture, larvae (especially the 4th through 7th instars) cause significant damage annually to large areas of pine trees during two disjunct periods (March to May, and then August to September) reducing stand volume and resin productivity. Flights of adult *D. kikuchii* usually occur in June/July and again in September/October, which overlap with the seasonal flight periods of the broadly sympatric species, *D. houi*, in Simao prefecture.

In heavily infested areas, *D. kikuchii* is controlled mainly by insecticide applications, the use of which could be enhanced and the environmental impact minimized by the timing of insecticide sprays by monitoring with pheromone traps (Zhang et al., 2003). So far, sex pheromones have been reported for the masson pine caterpillar moth, *D. punctatus* (Zhao et al., 1993), the pine moth, *D. spectabilis* (Ando et al., 1982; Kong et al., 2003), the larch caterpillar moth, *D. superans* (Klun et al., 2000; Kong et al., 2007a), the Yunnan pine caterpillar moth, *D. houi* (Kong et al., 2007b), and the European pine moth *D. pini* (Priesner et al., 1984; Kovalev et al., 1993). Sex pheromones components that have been identified from *Dendrolimus* species are all (5*Z*,7*E*)-5,7-dodecadienes with different terminal functional groups (alcohol, acetate, propionate, and aldehyde), except for the sex pheromone of *D. houi*, which was reported as a mixture of (5*E,*7*Z*)-5,7-dodecadien-1-yl acetate (*E*5,*Z*7-12:OAc), (5*E,*7*Z*)-5,7-dodecadien-1-ol (*E*5,*Z*7-12:OH), and (5*E*,7*Z*)-5,7-dodecadienal (*E*5,*Z*7-12:Ald) (Kong et al., 2007b). In preliminary GC analyses, appreciable amounts of *Z*5,*E*7-12:OAc were observed in extracts of pheromone glands of female *D. kikuchii*, along with smaller amounts of *Z*5,*E*7-12:OH (Kong et al., 2001). However, the identities of these compounds as pheromone components remained to be confirmed. In this study, we present additional analytical data on the pheromone gland components from female *D. kikuchii*, and report on their electrophysiological and behavioral activities in the laboratory and the field.

Methods and Materials

Collection and Handling of Insects Cocoons of *D. kikuchii* were collected from the host tree, *Pinus kesiya* var. *langbianensis,* near Pu'er city, Yunnan province and maintained in a rearing room at 25±1°C, 60-80% relative humidity with a 16L:8D photoperiod until adult emergence. Newly emerged males and females were housed separately under the same conditions. Virgin females were used for preparation of pheromone extracts, whereas males were used for electroantennographic studies.

Preparation of Samples Pheromone extracts were prepared as described previously (Kong et al., 2007a, b). Briefly, individual female sex pheromone glands were excised during the peak period of calling (0–2 d-old, 4–7 h into the scotophase) and extracted about 30 min in redistilled hexane (10 µl per gland). Glands were removed before sealing the extracts in glass tubes, which were stored at −20°C until bioassay and chemical analyses. Individual pheromone gland extracts were prepared (N=25) and 5 ng of (*Z*)-12-tetradecenyl acetate (*Z*12-14:OAc) were added to each as an internal standard to quantify the pheromone components.

Volatile pheromone collection was performed by solid phase microextraction (SPME) from virgin female moths (N=3) held in groups of 3-5 in 0.02 m^3 screen cages under ambient laboratory lighting and temperature conditions. After females were at rest on the walls of the cage for at least 30 min (to ensure quiescence during sample collection), we initiated collection of volatiles on a 70 µm SPME fiber (Supelco Inc., Bellefonte, PA, USA) coated with CarboWax/divinylbenzene (CW/DVB). The fiber was positioned close to the sex pheromone gland of a virgin female (2 mm) for the entire duration of the scotophase period. For analysis by GC-MS and GC-EAD, a 1 µl aliquot of a series of straight-chain aliphatic hydrocarbons (36 ng/µl of each in dichloromethane, see below for commercial source) was added directly to the sample-loaded fiber with syringe. The purpose of these standards was to assign Kováts retention indices (KI) to the components of the crude extract.

Chemical Standards Chemicals used in our analysis, e.g., all geometrical isomers of 5,7-dodecadien-1-yl acetate (*Z*5,*Z*7-, *E*5,*E*7-, *E*5,*Z*7-, and *Z*5,*E*7-12:OAc) and the corresponding alcohols (*Z*5,*Z*7-, *E*5,*E*7-, *E*5,*Z*7-, and *Z*5,*E*7-12:OH) and aldehydes (*Z*5,*Z*7-, *E*5,*E*7-, *E*5,*Z*7-, and *Z*5,*E*7-12:Ald), together with *Z*5-, and *E*5-12:OAc were purchased from Chemtech B. V. (Amsterdam, The Netherlands). *Z*12-14:OAc originated from our laboratory collection. All chemicals were purified by preparative GC using a 2 m×3.2 mm column (packed with 8% PEG 20 M on 80–100 mesh Chromosorb G-HP) in a Model 990 gas chromatograph (Perkin-Elmer Corp., Norwalk, CT, USA) as described by Kong et al. (2001, 2007b). Chemicals used as stimuli for electrophysiological and field trials were ≥99% chemically and ≥95% isomerically pure. C_{14}-C_{22} straight-chain hydrocarbons for assigning Kováts retention indices were purchased from TCI Co. (Tokyo, Japan). 4-Methyl-1,2,4-triazoline-3,5-dione (MTAD, 95%) was purchased from Aldrich Chemical (Milwaukee, WI, USA), and dimethyl disulfide (DMDS, 99%) and acetyl chloride (>99%) were purchased from Acros Organics (Geel, Belgium).

Derivatization Acetylation and methanolysis were performed to verify the conversion of alcohols (acetates) to the corresponding acetates (alcohols) (Bjostad et al., 1984). Positions of double bonds in conjugated dienes were determined by MTAD derivatization (Young et al., 1990; McElfresh and Millar, 1999), and in mono-unsaturated alkenes by the iodine-catalysed addition of dimethyl disulfide across the double bond (Buser et al., 1983). Derivatizations were performed in glass capillaries with one end sealed or in 1 ml conical vials with Teflon-lined screw caps. For acetylation and methanolysis, 14 female equivalents were evaporated to apparent dryness with a gentle nitrogen stream, then 20 µl acetyl chloride were added and allowed to react with extracted substances at room temperature for about 20 min. The product was again evaporated to dryness and re-dissolved in 6 µl of hexane. A 2 µl aliquot of the resulting solution was analyzed by GC with a flame ionization detector (FID). The remaining product of acetylation (4 µl) was evaporated again and 15 µl of KOH solution in methanol (0.5 M) were added. After 0.5 h at room temperature, 15 µl of a solution of HCl (1 M) and 30 µl hexane were added and the mixture was shaken for 2–3 min. Then 30 µl of redistilled water were added, the layers were separated and the recovered hexane portion was dried over anhydrous sodium sulfate for 24 h. After concentration with a stream of nitrogen, the solution was analyzed by GC-FID. For determination of double bond positions of conjugated dienes, MTAD derivatives of synthetic standards and compounds extracted from pheromone glands were prepared by adding 3 µl of 2 µg/µl MTAD in CH_2Cl_2 to gland extracts (7 female equivalents in 20 µl CH_2Cl_2) or to 10 ng of respective synthetic standard dissolved in 20 µl CH_2Cl_2. Mixtures were allowed to react at room temperature for 1 h. The products were concentrated and analyzed by GC-MS. For determination of double bond positions in mono-unsaturated acetates, synthetic standards and 14 female equivalent extracts in 20 µl hexane were derivatized following Buser's methods (Buser et al., 1983).

Electrophysiology The GC-EAD system consisted of a Hewlett-Packard 6890 GC equipped with flame ionization (FID) and electroantennogram (EAD) detectors. The EAD preparation consisted of a detached antenna from a 1-to 3-d-old male with the terminal segment removed, mounted directly between two EAG probes with electrically conductive gel (Spectra 360 electrode gel, Parker Laboratories Inc. Orange, NJ, USA). The signal from the EAD detector was amplified using a 10 × pre-amplifier and an AC/DC UN-6 amplifier (Syntech, The Netherlands) and fed to a personal computer equipped with an analog to digital conversion board (IDAC, Syntech). Acquisition and analysis of the EAD signals were performed by GC-EAD software (16-bit version, Syntech, The Netherlands). The GC was equipped with a split/splitless injector, a DB-WAX column (J&W Scientific, Folsom, CA, USA, 30 m× 0.25 mm i.d. × 0.25 µm) with the column effluent split by a splitter (SGE, part # OSS-2) between the FID and EAD detectors. The splitter (with a 12 ml/min N_2 make up gas to compensate for flow reduction) split the column effluent equally between the FID and EAD detectors, which were connected with the splitter by deactivated fused silica capillary columns (0.22 mm i.d.). The EAD arm was led out of the GC oven through a heated (250°C) EAD interface (Syntech) and the effluent exited into a purified and humidified airstream (~500 ml/min) directed over the antennal preparation. Synthetic compounds, solvent extracts, and SPME fibers with loaded samples were injected splitless using a temperature program starting at 80°C for 1 min, then programmed at 10°C /min to 200°C (20 min hold), N_2 was used as carrier gas, and the injector and detector temperature was set at 220°C and 250°C, respectively.

Gas Chromatography (GC) Products of acetylation and methanolysis were analyzed on an HP 5890 series II GC using a BP-20 capillary column (SGE analytical science, Brisbane, Australia, 25 m×0.22 mm i.d. × 0.25 µm) in splitless mode, temperature program: 80°C (1 min)-10°C/min-200°C (30 min). Quantitative analyses of 25 glands extracted individually were conducted on an HP6890 GC with a FID detector using a DB-WAX column (J&W Scientific, Folsom, CA, USA, 30 m×0.25 mm i.d.×0.25 µm) in splitless-mode, temperature program: 80°C (1 min)-6°C/min-200°C (10 min).

Coupled Gas Chromatography-Mass Spectrometry (GC-MS) Pheromone gland extracts and SPME samples (and all analyses for calculation of Kováts retention indices) were analyzed with a Finnigan Trace DSQ GC-MS (EI mode, 70 eV, mass range 41–460 amu) on DB-WAX and DB-5MS capillary columns (30 m×0.25 mm i.d. × 0.25 µm) with helium carrier gas (1.0 ml/min). SPME CW/DVB fibers were desorbed in the injection port for 2 min in splitless mode, program: 60°C (1 min)-6°C/min to 240°C (10 min hold), injector, ion source, and transfer line temperatures were set at 220°C, 250°C and 250°C, respectively. Full scan (TIC) and selected ion monitoring (SIM) spectra were recorded simultaneously. In SIM mode, diagnostic ions for the respective compounds were monitored. Thus, *m/z* 224 (M^+), 164 (M^+-CH_3COOH), and 136 ($C_{10}H_{16}^+$) were selected to characterize dodecadienyl acetates, *m/z* 182 (M^+), 164 (M^+-H_2O), and 121 (M^+-C_3H_9O) for dodecadienols, and *m/z* 166 (M^+-CH_3COOH) and 138 ($C_{10}H_{18}^+$) for dodecenyl acetates.

MTAD adducts were analyzed with a Finnigan Trace 2000 GC interfaced with a Voyager mass spectrometer fitted with a nonpolar HP-1 capillary column (50 m× 0.20 mm i.d.×0.33 µm; Agilent, J&W Scientific) in

splitless mode, program 80°C-15°C/min-280°C (20 min) with helium (0.6 ml/min) carrier gas in EI mode (70 eV), mass range from 41–401 amu, ion source, injector and transfer line temperatures 200°C, 280°C and 250°C, respectively. DMDS adducts were analyzed with a Finnigan Trace DSQ GC-MS (EI mode, 70 eV, mass range 41–560 amu) on a DB-5MS capillary column (30 m×0.25 mm i.d.×0.5 μm) in splitless mode. The column temperature was programmed as follows: 60°C (1 min)-6°C/min to 240°C (15 min hold). Injector, ion source, and transfer line temperatures were set at 250°C, 220°C, and 250°C, respectively. In GC-MS analysis, compounds were identified by comparison of retention indices and mass spectra with those of synthetic standards or their derivatives.

Field Behavioral Assays Lures consisted of gray rubber septa (The West Company, Phoenixville, PA, USA) loaded with hexane solutions (20 μl) of test compounds, plus 20 μl of butylated hydroxytoluene (BHT, 10 μg/μl) in dichloromethane as an antioxidant. Control septa were loaded only with 200 μg BHT in 20 μl dichloromethane. Once the treatment solutions had soaked into the septa, the lures were sealed in glass vials for transport. The lures were prepared 2 d before use and were mounted in the trap below the lid and about 2–3 cm above the sticky bottom. Experiments were set up in randomized, complete blocks with traps positioned approximately 20–30 m apart in a line and blocks separated by at least 150 m. Sticky traps, similar in shape to Pherocon 1C traps from Trécé Inc. (Salinas, CA, USA), were constructed from two pieces of cardboard (42×28 cm) with a wire in the center of the lid to suspend the septum. In traps baited with virgin females, a single virgin female moth that had emerged in the laboratory from field collected pupae was housed in a metal mesh container (8×6 cm) in place of the pheromone dispenser. Female moths were renewed every 4 d. Traps were fixed on pine tree branches ca. 1.5 m above ground, with the lures left unchanged for the entire flight period. Experiments in 2001 (eight treatments, four replicates) were designed to test male moth responses to *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH alone, and in binary combinations in different ratios. Experiments in 2007, 2009, and 2010 were designed to evaluate the effects of the trace compounds, *Z*5-12:OAc and *Z*5,*E*7-12:Ald, added to a base blend of *Z*5,*E*7-12:OAc/*Z*5,*E*7-12:OH in a 5:1 ratio. Male moths were removed and counted at 1-to 2-d intervals, depending on weather conditions during each study.

Data Handling and Statistical Analyses Kováts retention indices were calculated by using the formula described by Marques et al. (2000). For statistical analysis of field trials, the day counts for each treatment in a given block were pooled and this sum was transformed to log(x+1) to facilitate variance homogeneity prior to conducting one-way ANOVA. If significant treatment effects were detected ($P<0.05$), then the responses to the treatments were ranked by LSD *post hoc* tests ($\alpha=0.05$, SPSS13.0).

Results

GC-EAD Analyses In analyses of solvent extracts and SPME entrained volatiles from virgin female *D. kikuchii* moths by GC-EAD, up to three compounds that elicited responses from antennae of male moths were detected. The most active compound eluted at 19.33 min (Fig. 1, peak 2, Mean ± SD, 3.0±2.6 mV), and all male *D. kikuchii* antennae tested responded to this compound ($N=8$). The other two compounds that elicited antennal responses eluted earlier at 17.28 min (Fig. 1, peak 1, 0.3±0.6 mV, detected by 4 out of 8 antennae) and later at 21.09 min (Fig. 1, peak 3, 0.3±0.4 mV, detected by 3 out of 8 antennae). The GC retention times and EAD responses of the three compounds from glands agreed well with those of synthetic *Z*5-12:OAc (Mean ± SD, 2.3±1.7 mV, detected rate 100%, $N=6$), *Z*5,*E*7-12:OAc (4.0±2.9 mV, detected rate 100%, $N=6$), and *Z*5,*E*7-12:OH (0.1±0.1 mV, detected rate 33%, $N=6$), respectively, injected in 5 ng doses (2.5 ng at the antenna) during GC-EAD analyses. In addition, the antennae of male *D. kikuchii* also responded to synthetic *Z*5,*E*7-12:Ald and *E*5,*Z*7-12:Ald.

Quantitative analysis showed that single-gland extracts contained on average 1.2±0.9 ng (mean ng/female ± SD, $N=25$) of *Z*5,*E*7-12:OAc, 0.2±0.2 ng (mean ng/female ± SD, $N=25$) of *Z*5,*E*7-12:OH, and 0.01±0.05 ng (mean ng/female ± SD, $N=25$) of *Z*5-12:OAc corresponding to a ratio of 6:1:0.05.

GC-MS Analyses of Pheromone Extracts and MTAD and DMDS Adducts The first EAD-active compound EAD1 was characterized by retention indices on DB-WAX ($KI_{WAX}=1{,}909$) and DB-5 columns ($KI_{DB\text{-}5}=1{,}588$), respectively, with diagnostic ions of 166 (M^+-CH_3COOH, 14%), 138 ($C_{10}H_{18}^+$, 17%), 123 ($C_9H_{15}^+$, 11%), 110 ($C_8H_{14}^+$, 33%), and 67 ($C_5H_7^+$, 100%) (Tables 1, 2). The most abundant compound EAD2 had $KI_{WAX}=2{,}052$, and $KI_{DB\text{-}5}=1{,}636$, and was characterized by ions of *m*/*z* 224 (M^+, 4%), 164 (M^+-CH_3COOH, 23%), 136 ($C_{10}H_{16}^+$, 17%), 121 ($C_9H_{13}^+$, 28%), and 79 ($C_6H_7^+$, 100%). The third EAD-active compound EAD3 had $KI_{WAX}=2{,}149$, and $KI_{DB\text{-}5}=1{,}511$, respectively, and displayed ions at *m*/*z* 182 (M^+, 4%), 164 (M^+-H_2O, 17%), 135 (M^+-C_2H_7O, 6%), 121 (M^+-C_3H_9O, 19%), and 79 ($C_6H_7^+$, 100%). Mass spectra and retention times of the three EAD-active compounds

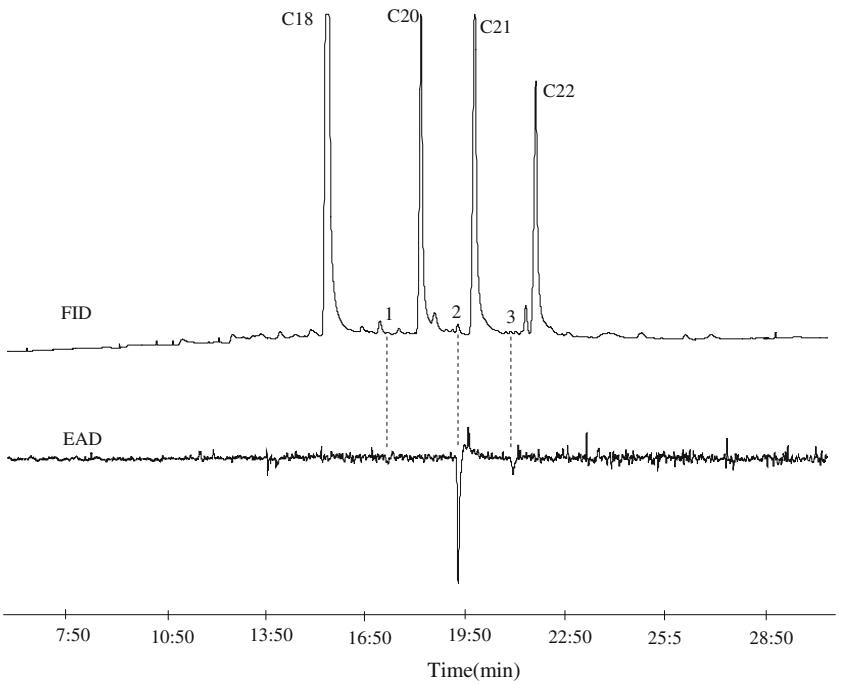


Fig. 1 Simultaneously recorded FID and EAD responses using antennae of male *Dendrolimus kikuchii* in response to sample collected by the CW/DVB fiber from one virgin female *D. kikuchii* moths. Peaks indicated by 1, 2, and 3 represent *Z*5-12:OAc, *Z*5,*E*7-12:OAc, and *Z*5,*E*7-12:OH, respectively. A 1 μl solution of hydrocarbon standards (36 ng/μl each) was added onto the fiber before injection

were identical to those of *Z*5-12:OAc (EAD1), *Z*5,*E*7-12:OAc (EAD2), and *Z*5,*E*7-12:OH (EAD3), respectively (Tables 1, 2), confirming the presence of the three compounds in the pheromone glands of *D. kikuchii*. Identities of the EAD2 and EAD3 compounds were further confirmed by microchemical analyses. Mass spectra and retention times of MTAD derivatized compounds in the gland extracts were identical to those obtained by MTAD treatment of synthetic *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH (Table 1). Conjugated double bond positions of the EAD2 and EAD3 compounds were 5,7 based on examination of diagnostic fragments from cleavage on either side of the adducts formed between the gland components and the dienophile MTAD (Table 1). In addition, acetylation and

Table 1 Electron impact mass spectral data from synthetic standards, components of pheromone gland extracts, and their MTAD and DMDS derivatives for the Simao pine caterpillar, *Dendrolimus kikuchii*

Components/Derivatives	Ions, *m/z*/abundance, % [a]				
*Z*5,*E*7-12:OAc					
Standard	224[b] /4	164[b] /20	136[b] /17	121/29	79/100
Solvent extracts	/3	/19	/16	/24	/100
SPME collections	/4	/23	/17	/28	/100
*Z*5,*E*7-12:OH					
Standard	182[b] /4	164[b] /19	135/7	121[b] /19	79/100
Solvent extracts	/4	/18	/7	/21	/100
SPME collections	/4	/17	/6	/19	/100
*Z*5-12:OAc					
Standard	166[b] /14	138[b] /18	123/9	110/35	67/100
Solvent extracts	/12	/11	/7	/22	/100
SPME collection	/14	/17	/11	/33	/100
Derivatives					
*Z*5,*E*7-12:OAc + MTAD	337/8	280/21	223/14	222/100	165/26
Solvent extracts + MTAD	/8	/21	/14	/100	/28
*Z*5,*E*7-12:OH + MTAD	295/8	238/26	222/100	181/13	165/24
Solvent extracts + MTAD	/9	/25	/100	/11	/24
*Z*5-12:OAc + DMDS	320/41	273/2	175/15	145/100	115/41
Solvent extracts + DMDS	/31	/2	/12	/100	/53

[a] Full-scan spectra obtained. MTAD, 4-methyl-1,2,4-triazoline-3,5-dione. DMDS, dimethyl disulfide

[b] Selected ion monitoring spectra obtained from solvent extracts of pheromone glands

Table 2 Compounds, Kováts retention index values, and relative quantities of pheromone candidates from calling virgin female *Dendrolimus kikuchii* analyzed by GC-MS with both DB-WAX and DB-5MS capillary columns

Compound	Kováts retention index					Relative quantity (%)		
	DB-WAX column			DB-5MS column		DB-WAX column		DB-5MS column
	standard	SPME extracts	Solvent extracts	Standard	Solvent extracts	SPME samples (Mean ± SE, *N*=3)	Solvent extracts (Mean ± SE, *N*=6)	Solvent extracts (Mean ± SE, *N*=5)
*Z*5,*E*7-12:OAc	2,052	2,052	2,052	1,636	1,636	100	100	100
*E*5,*Z*7-12:OAc	2,062	nd	nd	1,640	nd	nd	nd	nd
*Z*5,*Z*7-12:OAc	2,077	2,076	2,077	1,653	nd	0.64±0.52	0.39±0.22	nd
*E*5,*E*7-12:OAc	2,097	2,096	nd	1,666	nd	2.02±1.65	nd	nd
*Z*5,*E*7-12:OH	2,149	2,148	2,149	1,511	1,511	7.18±0.83	17.82±4.49	4.03±2.54
*E*5,*Z*7-12:OH	2,156	nd	nd	1,514	nd	nd	nd	nd
*Z*5,*E*7-12:Ald	1,875	1,874	1,874	1,436	nd	0.82±0.66	1.78±1.02	nd
*E*5,*Z*7-12:Ald	1,886	nd	nd	1,441	nd	nd	nd	nd
*Z*5-12:OAc	1,909	1,909	1,909	1,588	nd	1.01±0.83	0.57±0.33	nd
*E*5-12:OAc	1,914	nd	nd	1,591	nd	nd	nd	nd

nd not detected

base methanolysis of gland extracts resulted in disappearance and re-appearance of the alcohol peak, confirming unambiguously the OH moiety in the EAD3 compound. The mass spectral data of compounds in the DMDS derivatized gland extracts of *D. kikuchii* (Table 1) indicated the presence of the double bonds in the Δ^5 position (*m*/*z* 175 and 145) for the minor EAD1 components. The retention times matched those of the corresponding derivative from *Z*5-12:OAc, thus confirming the previous identifications. The other 5,7-isomers were excluded from consideration because their retention times were different from those of the *Z*5,*E*7-compounds. These data, along with the MTAD data, would completely prove the double bond positions and stereochemistries.

Field Behavioral Assays Preliminary field trials in 2001 had indicated that neither *Z*5,*E*7-12:OAc nor *Z*5,*E*7-12:OH was attractive alone and must be presented together (Table 3). Lures with higher doses of *Z*5,*E*7-12:OAc than *Z*5,*E*7-12:OH (9:1, 3:1 ratios) showed better attractiveness than the blends with opposite ratio (Table 3). Field experiments performed in 2007 showed that there was no significant difference in male moth captures to *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH at different ratios tested (Table 3).

Increasing the amount of *Z*5-12:OAc from 1% to 25% of the major component, *Z*5,*E*7-12:OAc, suggested an optimum level of 25% in trap captures relative to the acetate/alcohol blend (5:1 ratio) (Table 4). The same field trials conducted in 2009 and 2010 confirmed the results; the most attractive blend contained these three components in a 100:20:25 ratio released from a gray rubber septum (Table 4). The optimum synthetic pheromone blend caught more than twice as many male *D. kikuchii* moths as traps baited with a single virgin female moth (Table 4). Addition of 20 μg *Z*5,*E*7-12:Ald to either the binary blend (*Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH) or ternary blend (*Z*5,*E*7-12:OAc, *Z*5,*E*7-12:OH, and *Z*5-12:OAc) resulted in significant reductions in trap catches relative to the acetate/alcohol blend or the optimum 3-component blend (Table 4).

Table 3 Catches of male *Dendrolimus kikuchii* in traps baited with various combinations of *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH, Yunnan province, China

Composition of baits (μg)		Mean catch ± SE / trap [a] *N*=4
*Z*5,*E*7-12:OAc	*Z*5,*E*7-12:OH	
Experiment (2001)		
1000	0	0
0	1000	0
900	100	2.75±0.85 ab
750	250	7.00±2.61 a
500	500	0.75±0.48 bc
250	750	0.25±0.25 c
100	900	0
control		0
Experiment (2007)		
450	150	8.00±1.41 a
500	100	7.00±1.00 a
525	75	5.00±0.58 a
control		1.50±0.96 b

[a] Means followed by the same letter are not significantly different at the 5% confidence level by LSD tests ($F_{(3,12)}$=8.10, P<0.003 for Experiment 2001 on June 19-July 2 in Zhenyuan county; $F_{(3,12)}$=7.35, P<0.005 for Experiment 2007 on June 16–30 in Pu'er city)

Table 4 Catches of male *Dendrolimus kikuchii* in traps baited with various blends of potential pheromone components in Pu'er city, Yunnan province, China

Composition of baits (μg)				Mean catch ± SE/trap[a]		
*Z*5,*E*7-12:OAc	*Z*5,*E*7-12:OH	*Z*5-12:OAc	*Z*5,*E*7-12:Ald	Experiment (2007) *N*=4	Experiment (2009) *N*=5	Experiment (2010) *N*=4
500	100	0	0	7.00±1.78 ab	3.80±0.73 bc	4.50±1.32 bc
500	100	5	0	1.75±0.48 cd	2.60±0.68 c	1.50±0.61 c
500	100	25	0	5.00±2.42 bc	5.20±1.16 b	3.00±0.58 bc
500	100	125	0	11.00±1.29 a	9.80±1.66 a	16.50±3.77 a
500	100	250	0	–[b]	–	9.00±3.24 ab
500	100	125	20	–	0	0
500	100	0	20	1.00±0.34 d	0.80±0.37 d	–
Live female				–	4.20±0.86 bc	6.00±2.27 ab
Control				1.50±0.65 d	0	0

[a] Means followed by the same letter in each column are not significantly different at the 5% confidence level by LSD tests (Experiment 2007, June 12-July 8, $F_{(5,18)}=10.52$, $P<0.001$; Experiment 2009, June 16–30, $F_{(5,24)}=10.83$, $P<0.001$; Experiment 2010, June 11–23, $F_{(5,18)}=4.05$, $P<0.01$)

[b] "–" indicated the treatments were not conducted

Discussion

Two suspected pheromone components, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH, have been known from *D. kikuchii* moths for quite some time but their functions were unclear (Kong et al., 2001). The present study describes efforts to clarify the female-produced sex pheromone of *D. kikuchii*, which apparently consists of at least three compounds, i.e., the primary component *Z*5,*E*7-12:OAc and the less abundant *Z*5,*E*7-12:OH and *Z*5-12:OAc. These three compounds were detected in both pheromone gland extracts and SPME entrained volatiles of calling females by GC-MS and GC-EAD methods. The antennae of male *D. kikuchii* were most sensitive to *Z*5,*E*7-12:OAc followed by *Z*5-12:OAc and *Z*5,*E*7-12:OH in GC-EAD analyses, although *Z*5-12:OAc is 20-fold less abundant than *Z*5,*E*7-12:OH in gland extracts. Following the common pattern of pheromone chemistry in the *Dendrolimus* genus, female moths of *D. kikuchii* employ the *Z*5,*E*7-configuration of the acetate and alcohol pheromone components and both components are essential for attraction. In this study, field trapping data also have provided some evidence for the behavioral role of the minor EAD-active component, *Z*5-12:OAc, confirming its status as a pheromone component of *D. kikuchii*. In addition, *Z*5-12:OAc also has been found in *D. punctatus*, although it is not part of the attractive pheromone blend of that species (Zhao et al., 1993).

SPME samples obtained by CW/DVB coated fiber (70 μm) and conventional samples obtained by solvent extraction were similar, and provided enough material for GC-MS analyses of the most abundant *Z*5,*E*7-12:OAc. Clear mass spectra were not obtained from solvent extracts of *Z*5,*E*7-12:OH and *Z*5-12:OAc because the compounds were present in much smaller quantities than in the SPME samples. SPME sampling was more effective and provided enough material for unambiguous full scan GC-MS identification. The assignments of the stereoisomers of the EAD-active peaks were completed by comparison of their retention indices with those of the synthetic acetate and alcohol on DB-WAX and DB-5MS columns. The retention times on two columns of different polarity provide strong evidence of the identity of the three compounds. In addition, the GC-MS data of MTAD and DMDS derivatization with gland extracts further confirmed the presence of *Z*5,*E*7-12:OAc, *Z*5,*E*7-12:OH, and *Z*5-12:OAc in the pheromone glands. The fact that the retention times of the other 5,7 isomers were different also confirms the stereochemistry as *Z*5,*E*7.

Female-emitted sex pheromones typically have two or more components, and component ratio often is important in evoking optimal responses from males (Linn and Roelofs, 1989). Although the response of *D. kikuchii* males broadly overlaps the component ratios found in the gland extracts of virgin females with different methods, in the species studied here, the pheromone component ratios are important as in the closely related *D. punctatus* and *D. spectabilis*, which are sympatric in northern China. *Dendrolimus kikuchii* and *D. spectabilis* are entirely allopatric and so can use *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH as major pheromone components, *D. kikuchii* in a 100:7 (SPME) ratio and *D. spectabilis* in about the inverse ratio (Kong et al., 2003). Over much of southern China, *D. kikuchii* is broadly sympatric with *D. punctatus*, which employs a 100:75 blend of *Z*5,*E*7-12:OH and *Z*5,*E*7-12:OAc as major pheromone components (Zhao et al., 1993).

The difference in ratios between major pheromone components could serve as a premating reproductive isolation mechanism between the sympatric *D. kikuchii* and *D. punctatus*. Furthermore, the minor components, *Z*5-12:OAc and (5*Z*,7*E*)-5,7-dodecadien-1-yl propionate, function to enhance the specificity of the signals, respectively. In addition, *Z*5,*E*7-12:OAc is known to serve as a strong inhibitor of European pine caterpillar moth *D. pini* whose major pheromone component is *Z*5,*E*7-12:Ald (Priesner et al., 1984). In the current research, *Z*5,*E*7-12:Ald at relatively high dose showed strong antagonistic effects on attraction of male *D. kikuchii*, although trace quantities of it have been found in the gland extracts. From the point of electrophysiological evidence, *Z*5,*E*7-12:Ald and *E*5,*Z*7-12:Ald might be involved in the inhibition of sympatric species *D. kikuchii* and *D. houi*.

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Rapid Identification of Insect Cuticular Hydrocarbons Using Gas Chromatography—Ion-Trap Mass Spectrometry

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Abstract Insect cuticular hydrocarbons (CHCs) are not only essential for desiccation resistance, they also play an important role as chemical signals and cues in social as well as solitary insects. The identification of CHCs is, therefore, crucial to an understanding of the chemical communication within and between insect species. We describe a method for rapid, simple, and unambiguous identification of CHCs using gas chromatography—ion-trap mass spectrometry. External ionization configuration in combination with a low ion-trap temperature resulted in dramatically increased intensities of molecular ions for alkanes, alkenes and alkadienes, and in high-mass fragmentation patterns with intense ions characteristic for methyl-branched hydrocarbons comparable to those obtained with quadrupole instruments. Additionally, we present an external chemical ionization—tandem mass-spectrometric method that allows for the determination of double-bond positions in alkenes and alkadienes without the need for derivatization prior to analysis.

Key Words Cuticular hydrocarbons · Chemical ionization · Ion-trap · Tandem mass spectrometry · *Drosophila melanogaster* · *Cerceris arenaria*

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Introduction

Cuticular hydrocarbons (CHCs) play a key role for the ecology and chemical communication in solitary and social insects and have, therefore, been subject to extensive study (e.g., Ferveur, 2005; Howard and Blomquist, 2005; Le Conte and Hefetz, 2008; Martin and Drijfhout, 2009). In addition to their importance for desiccation resistance, CHCs can serve as pheromones (i.e., sex, trail, queen, recruitment, dominance pheromones) that confer information in a diverse range of contexts to con- or heterospecific individuals. Insect CHCs comprise simple to very complex mixtures of saturated, unsaturated, and methyl-branched hydrocarbons.

Although many CHCs can be analyzed by using matrix-assisted laser desorption/ionization (MALDI) or laser desorption/ionization time-of-flight (LDI-TOF) mass spectrometry (Cvacka et al., 2006; Yew et al., 2009), most studies use gas chromatography—mass spectrometry (GC-MS) for routine identification of these substances. Single-quadrupole analyzers have been used most frequently for these studies due to ease of use, availability, and simple interpretation of mass spectra (e.g., molecular ions of alkanes and alkenes can readily be identified, characteristic fragmentation patterns of methyl-branched hydrocarbons; e.g. Nelson et al., 1972; Pomonis et al., 1978). Determination of double-bond positions in alkenes usually is accomplished by dimethyldisulfide (DMDS) derivatization followed by GC-MS analysis (Dunkelblum et al., 1985). However, due to retention time shift and the high boiling point of derivatized long-chained alkenes, determination of double-bond positions is limited to hydrocarbons of medium molecular weight, and assignment of derivatized compounds to the corresponding alkene may be difficult.

Compared to single-quadrupole analyzers, ion-trap mass spectrometers rarely have been used for the identification of

CHCs, despite the fact that they offer many advanced features such as tandem mass spectrometry (MS-MS) and various chemical ionization (CI) techniques that make them the instrument of choice for many other applications. The rare use of ion-trap instruments is due mainly to differential fragmentation patterns of methyl-branched hydrocarbons (Doolittle et al., 1995) and low abundance of molecular and characteristic fragment ions in internal ionization configuration, which can be partially overcome by a low ion-source temperature and increased ionization time in high-mass segments (Doolittle et al., 1995). However, these measures may have certain disadvantages, e.g., increased detector tailing and elevated background ions. Recent advances in ion-trap instrumentation, especially the use of external ionization, combine the advantages of both quadrupole and ion-trap technologies. Briefly, in internal ionization configuration, both the GC transfer line and the CI gas transfer line terminate directly in the ion-trap, and ionization occurs in the trap itself. In external ionization configuration, however, the GC transfer line and the CI gas transfer line end in an external ion source, where the sample is ionized and then directed towards the ion-trap assembly by ion lenses using electrostatic focusing.

Here, we report on the optimization of the ion-trap temperature of a GC ion-trap mass spectrometer in external ionization mode for the identification of alkanes, methyl-branched alkanes, alkenes, and alkadienes in combination with a CI MS-MS *in situ* double bond derivatization method. This procedure allows for the unambiguous, simple, and rapid identification of insect CHCs, including the determination of the double-bond position(s), without the need of pre-analysis chemical derivatization.

Methods and Materials

GC-MS GC-MS analyses were performed using a Varian 240MS ion-trap mass detector coupled to a Varian 450GC. All analyses were performed in the external ionization configuration. A VF-5 ms column (30 m×0.25 mm ID; 0.25 μm df; Varian Inc. Palo Alto, CA, USA) was used to separate the hydrocarbons. The temperature of the split/splitless GC injector was set to 250°C, and the injector was operated in the split [electron ionization (EI)] or splitless mode [chemical ionization (CI)], respectively. The GC oven temperature was programmed as follows: 150°C for 1 min, 5°C/min to 300°C, hold 5 min. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. EI spectra were recorded with a mass range of *m/z* 45–500 at maximum scan rate. CI spectra were recorded using acetonitrile (ACN) as a reagent gas with a mass range of *m/z* 60–500. In ACN-CI, the diagnostically useful $[C_3H_4N]^+$ ion is formed from an ion/molecule reaction between $[C_2H_2N]^+$ (*m/z* 40) and neutral acetonitrile, followed by a loss of HCN (Oldham, 1999). CI MS-MS experiments of alkene $[M+54]^+$ adducts (Moneti et al., 1997; Oldham and Svatos, 1999) were conducted with the resonant waveform type for precursor ion excitation, an isolation window of *m/z*=5, and 20 ms excitation time. The automatic "q" calculator was used for the determination of precursor ion excitation energy and product ion mass range. CI MS-MS experiments of alkadienes were conducted in the same fashion as for the alkenes with the $[M+54]^+$ adduct serving as the precursor ion. All CI measurements were conducted with an ion-trap temperature of 90°C, a manifold temperature of 40°C, and an ion-source temperature of 160°C. Data were analyzed using the MS Workstation software (version 6.9.3, Varian Inc., Palo Alto, CA, USA).

Influence of Ion-trap Temperature on Characteristic Ion Abundance in EI Mode The influence of ion-trap temperature on the abundance of alkane, alkene, and alkadiene molecular ions as well as "classical" fragment ions of methyl-branched alkanes in the external ionization EI mode was assessed by varying the ion-trap temperature from 80°C to 160°C in 10°C-steps. The ion-source temperature was always kept at 160°C. The ion-trap temperature was not lowered below 80°C to avoid excess temperature differences between the ion source and the ion-trap. The manifold temperature was kept at 40°C (for ion-trap temperatures between 80°C and 120°C) or at 50°C (for ion-trap temperatures between 130°C and 160°C), respectively. After each temperature change, the ion-trap radio frequency ramp was adjusted and the instrument was tuned in EI mode using the auto-tune function. At each ion-trap temperature step, an alkane standard solution containing all alkanes from C_8 to C_{40} with a concentration of 40 ng/μl for each compound (composed from alkane solutions C_8-C_{20} (Fluka) and C_{21}-C_{40} (Fluka)), a CHC extract of a *Cerceris arenaria* female, and a CHC extract of *Drosophila melanogaster* females (see below) were injected into the GC-MS in EI mode.

Insects and Sampling Females of the solitary digger wasp *Cerceris arenaria* (Hymenoptera, Crabronidae) were caught in a sand pit near Alzenau, Germany. The wasps were anesthetized with CO_2, frozen at −20°C, and individually extracted in 1 ml of hexane for 10 min. CHCs of this species have been identified previously using a GC quadrupole MS, and the position of alkene double-bonds has been determined using DMDS derivatization followed by GC-MS analysis (Strohm et al., 2008). *Drosophila melanogaster* (Diptera, Drosophilidae) was used as a second model organism to study the suitability of the CI

MS/MS method for the determination of the double-bond positions in alkadienes. CHCs of this species have been studied extensively (e.g., Everaerts et al., 2010), and double-bond positions of alkadienes have been described. For CHC extraction, 490 6-d-old virgin *D. melanogaster* females (wt, Berlin) were deposited in a Pasteur glass pipette, stopped on the narrower end with pre-extracted glass wool, and gradually washed with hexane (2.45 ml; Aldrich) to yield one female equivalent per 5 µl of solution.

Data Analysis The abundance of molecular ions of alkanes was calculated for the C_{21} to C_{31} alkanes from the alkane standard solution. This represents the range of alkanes that are most commonly found in insects. The abundance of molecular ions of alkenes was calculated from five naturally occurring alkenes (from *C. arenaria* and *D. melanogaster)*: (*Z*)-7-tricosene, (*Z*)-7-pentacosene, (*Z*)-7-heptacosene, 7-nonacosene, 7-hentriacontene (Strohm et al., 2008). The abundance of alkadiene molecular ions was calculated from (*Z*,*Z*)-7,11-tricosadiene, (*Z*,*Z*)-7,11-pentacosadiene, (*Z*,*Z*)-7,11-heptacosadiene, and (*Z*,*Z*)-7,11-nonacosadiene of the *D. melanogaster* extract (Everaerts et al., 2010). The abundance of "classical" diagnostic fragment ions of methyl-branched alkanes (α-cleavage) was calculated from six selected cuticular compounds of *C. arenaria* (7-methylpentacosene = 7me-C25 (*m/z* 281), 5me-C25 (*m/z* 309), 3me-C25 (*m/z* 337), 7me-C27 (*m/z* 309), 5me-C27 (*m/z* 337), and 7me-C29 (*m/z* 337)). Although internally branched monomethyl-alkanes and dimethyl-alkanes also yield good mass spectra, we used terminally branched hydrocarbons for the calculation of ion abundances for two reasons. First, internally branched hydrocarbons are chromatographically difficult to separate (Carlson et al., 1998) and, therefore, calculation of ion abundances is hampered by the coelution of several different compounds resulting in a mixed mass spectrum. Second, if the methyl-group is located in the middle of a molecule, both characteristic ions resulting from the cleavages at the methyl-group occur in similar (intermediate) abundances, and are less prominent than the more abundant ion in terminally branched alkanes. The only exceptions are symmetric molecules (e.g., 13me-C25). This behavior, however, also is observed in quadrupole instruments. Therefore, the effect of the ion-trap temperature on the abundance of characteristic ions of methyl-branched alkanes was assessed from terminally branched alkanes.

All abundances are expressed as the percentage of the base peak (*m/z* 57 for alkanes and methyl-alkanes, *m/z* 55 for alkenes, and *m/z* 67 for alkadienes). We compared abundances of the diagnostic ions of all compound classes among different ion-trap temperatures using a Friedman test. The statistical analyses were conducted using SPSS 17.0.0 software.

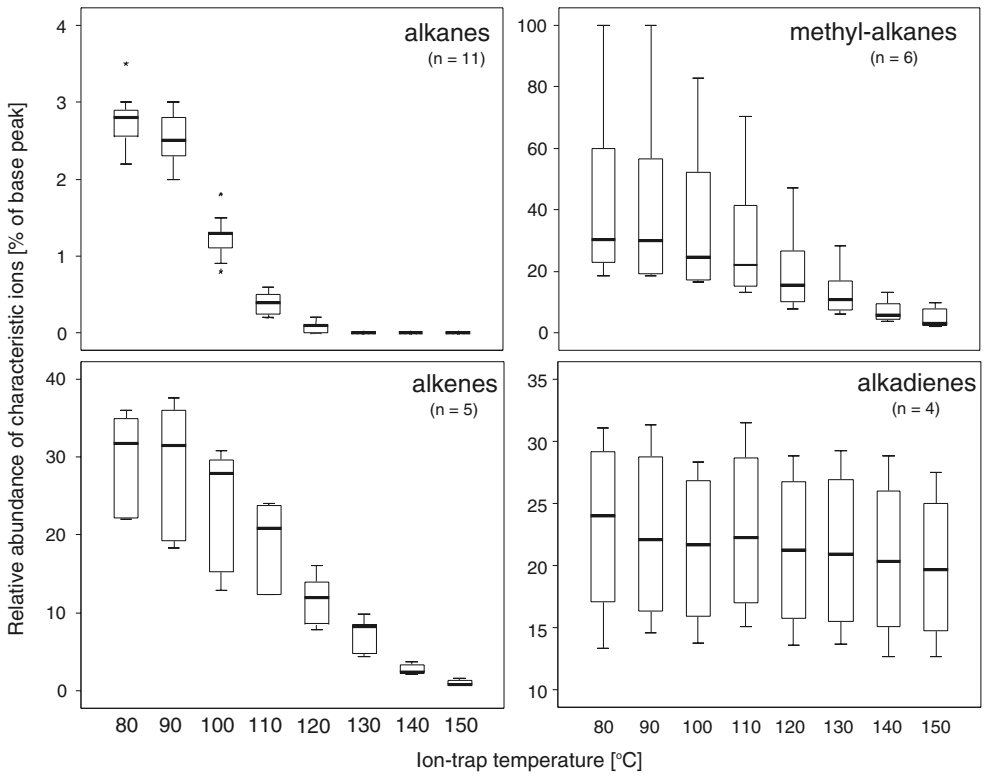


Fig. 1 Influence of ion-trap temperature on the relative abundance of the molecular ions of alkanes, alkenes, and alkadienes, as well as characteristic fragment ions of methyl-branched alkanes in external EI configuration (in percent of the base peak). *Bold lines* represent medians, *boxes* comprise the interquartile ranges, *bars* indicate minimum and maximum values, and *asterisks* represent outliers

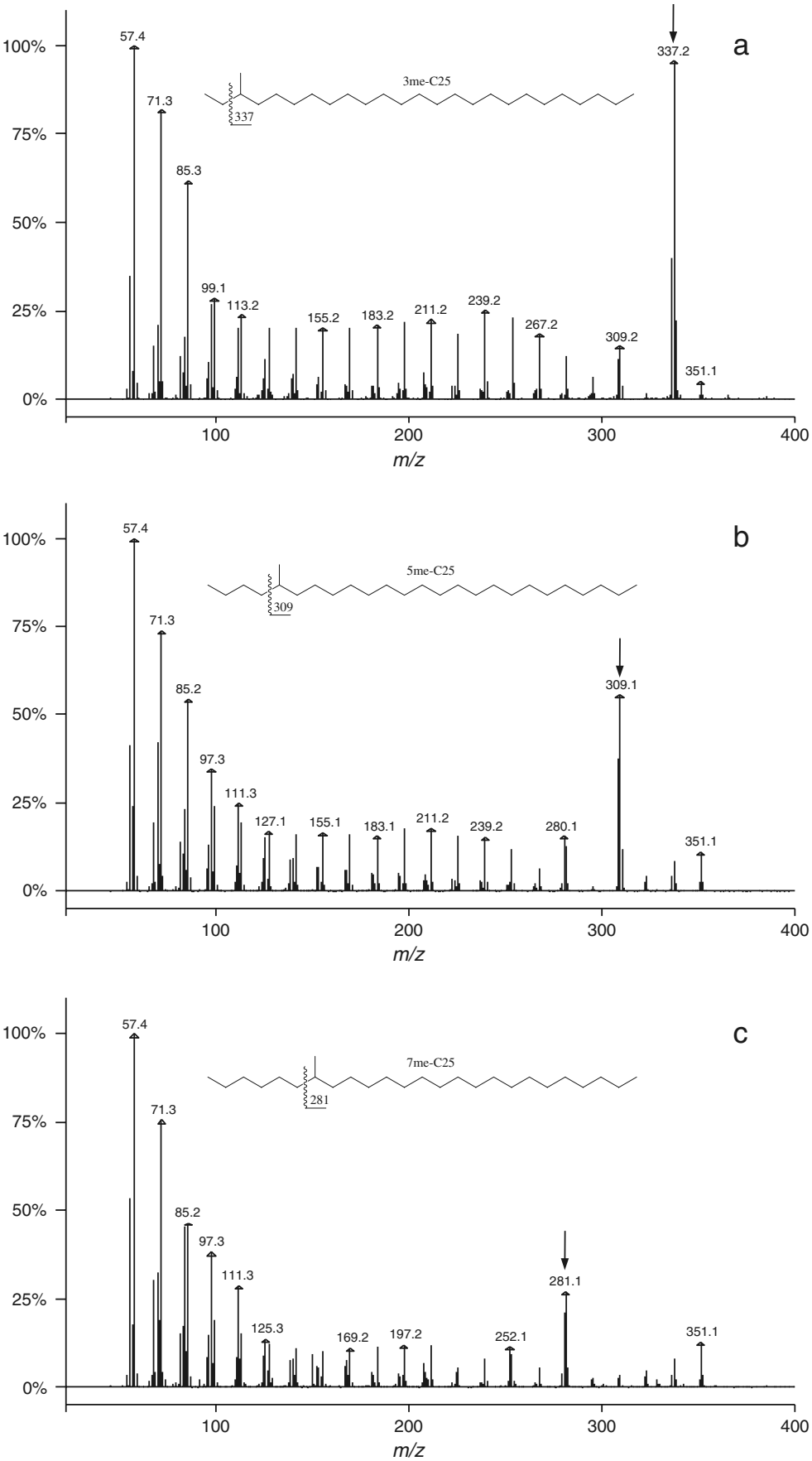


Fig. 2 Electron ionization (EI) mass spectra of different isomers of methyl-pentacosane (*m/z* 366) from a cuticular extract of *Cerceris arenaria* as measured at an ion-trap temperature of 80°C. **a** 3-methyl-pentacosane (3me-C25); **b** 5-methyl-pentacosane (5me-C25); and **c** 7-methyl-pentacosane (7me-C25). Formation of the most abundant characteristic ion by α-cleavage is given for each isomer, and this characteristic ion is indicated in the mass spectrum by an *arrow*, respectively

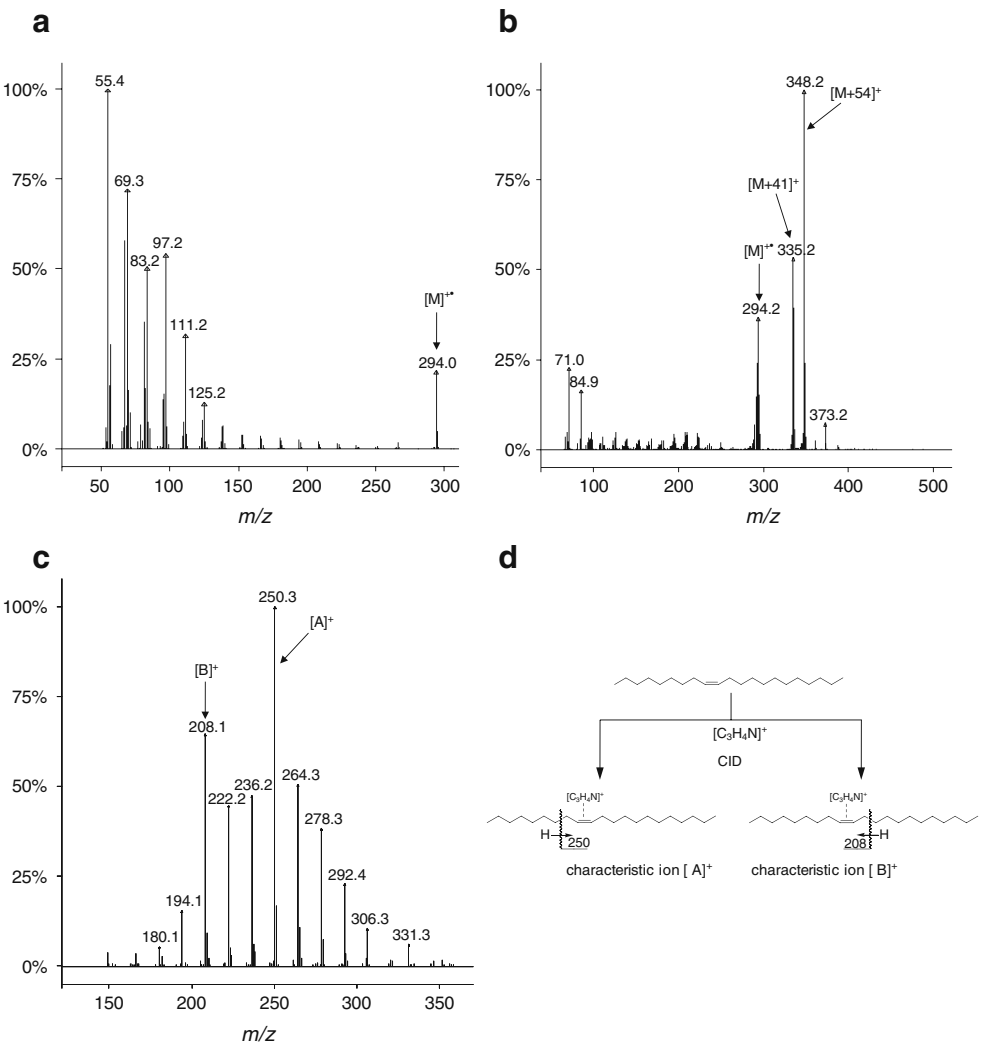


Fig. 3 Mass spectra of synthetic (*Z*)-9-heneicosene after **a** electron ionization (EI) at 80°C ion-trap temperature; **b** chemical ionization (CI) with acetonitrile; **c** acetonitrile-CI MS/MS of the $[M+54]^+$ adduct ion; and **d** observed fragmentation pattern upon collision-induced dissociation (CID) in the ion-trap MS/MS experiment. Molecular ions ($[M]^{+\bullet}$, *m/z* 294), acetonitrile adduct ions (*m/z* 335 and *m/z* 348, representing $[M+41]^+$ and $[M+54]^+$, respectively), and characteristic fragment ions after MS/MS (*m/z* 250 and *m/z* 208, representing fragment ions $[A]^+$ and $[B]^+$, respectively) are indicated by *arrows*

Results

In EI mode, we observed a strong influence of the ion-trap temperature on the abundance of molecular ions for alkanes (Friedman test: $N=11$, $P<0.001$) and alkenes (Friedman test: $N=5$, $P<0.001$). The influence on abundances of molecular ions of alkadienes was smaller, but still significant (Friedman test: $N=4$, $P<0.01$). Lower ion-trap temperature resulted in a significant increase of molecular ion intensity with a maximum at 80°C (Fig. 1). Alkenes and alkadienes generally had more abundant molecular ions than alkanes.

In contrast to previous reports that used ion-trap instruments in the internal ionization mode (Doolittle et al., 1995), our use of external ionization yielded σ-cleavages and hydrogen transfer reactions characteristic for fragmentation of methyl-branched hydrocarbons comparable to those observed in quadrupole or sector-field instruments (Fig. 2). As for the molecular ions of linear alkanes, the abundance of diagnostic fragment ions was strongly dependent on the ion-trap temperature, with a lower temperature resulting in a higher abundance of diagnostic ions (Friedman test: $N=6$, $P<0.001$; Fig. 1). The strongest diagnostic ions were observed at the lowest ion-trap temperature tested (80°C). As in quadrupole MS, terminally branched CHCs yielded much stronger diagnostic ions than internally branched CHCs at low ion-trap temperatures (Fig. 2).

For the determination of the double-bond positions in alkenes and alkadienes, we applied a CI MS/MS approach using acetonitrile as a reagent gas. For alkenes, CI gave strong $[M+41]^+$ and $[M+54]^+$ adduct ions (Fig. 3b). The molecular ions also were easily visible. We did not find, however, the diagnostic *j*- and *l*-ions (cleavage at the position of the former double bond), described by Moneti et al. (1997) under internal ionization conditions. MS/MS experiments using the $[M+54]^+$ adducts as fixed precursor ions resulted in fragmentation at the (former) allylic positions (Fig. 3c and d). This finding is congruent with the pattern described by Oldham and Svatoš (1999) for CI

with ACN under internal ionization, and a reaction level of 50 (only reagent gas ions > *m/z* 50 react with the analyte). With increasing chain length of alkenes, the intensity of the respective small diagnostic ion decreased markedly. The larger ion became more prominent, and allowed for the unambiguous determination of the double-bond position. This dominance of the larger fragment ion is in congruence with Stevenson's rule, which dictates that the more stable carbocation (in this case, the larger fragment) is able to keep the charge more effectively. Formation of the larger ion is favored, and this ion will be more abundant in the mass spectrum. In addition to the characteristic ions, a whole series of ions spaced by 14 u (CH_2-moieties) are detectable, which is due to the stochastic nature of the fragmentation process that also allows less probable ions to be formed, albeit in lower quantities.

For alkadienes, CI using ACN as the reagent gas resulted in strong $[M+54]^+$ adduct ions. However, the $[M+41]^+$ adduct ion peak was much less pronounced (Fig. 4b). CI MS/MS experiments using the $[M+54]^+$ adduct ions as precursor ions gave two characteristic fragments with cleavages at the outward α-position of the two respective double bonds (Fig. 4c and d). This observation agrees with results of a previous ACN CI MS/MS study performed on diunsaturated fatty acids methyl esters, which also proposed a mechanism for the formation of the characteristic ions by cycloaddition of the ACN $[M+54]^+$ ion across the double bond, followed by Cope rearrangement and a retro-ene reaction after collision-induced dissociation (Van Pelt et al., 1999).

Discussion

Our experiments show that GC ion-trap mass spectrometry offers an excellent possibility for rapid and uncomplicated analysis of insect CHCs. External ionization in combination with low ion-trap temperature yields a sufficient abundance of diagnostic ions of alkanes,

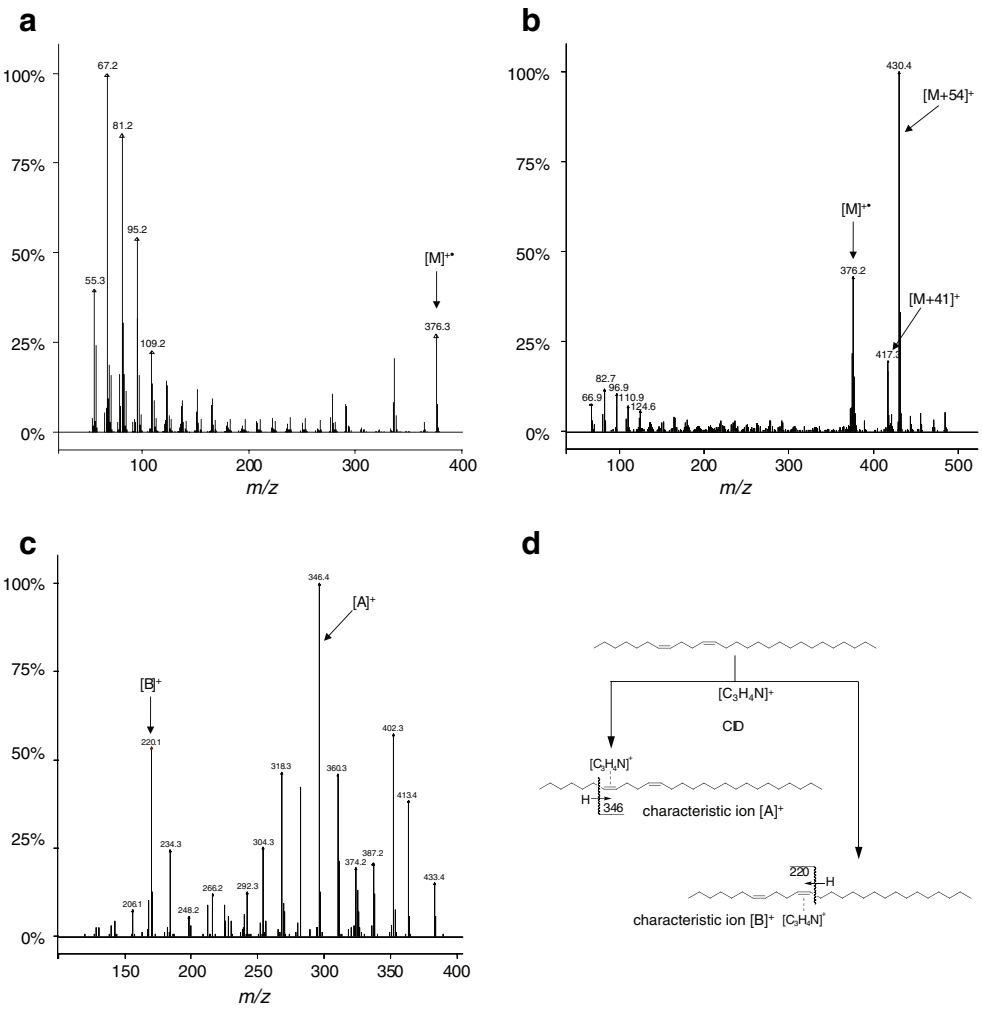


Fig. 4 Mass spectra of (*Z*,*Z*)-7,11-heptacosadiene from a cuticular extract of *Drosophila melanogaster* after **a** electron ionization (EI); **b** chemical ionization (CI) with acetonitrile; **c** acetonitrile-CI MS/MS of the $[M+54]^+$ adduct ion; and **d** observed fragmentation pattern upon collision-induced dissociation (CID) in the ion-trap MS/MS experiment. Molecular ions ($[M]^{+\bullet}$, *m/z* 376), acetonitrile adduct ions (*m/z* 417 and *m/z* 430, representing $[M+41]^+$ and $[M+54]^+$, respectively), and characteristic fragment ions after MS/MS (*m/z* 346 and *m/z* 220, representing fragment ions $[A]^+$ and $[B]^+$, respectively) are indicated by *arrows*

alkenes, and methyl-branched alkanes comparable to quadrupole and sector-field instruments. The dramatic effect observed for alkenes and linear and methyl-branched alkanes indicates that keeping the trap at high temperatures reduces lifetime of the molecular and high-mass diagnostic ions, thus resulting in the widespread prejudice against using ion-traps for CHC analysis. Molecular ions of polyunsaturated CHCs, however, are more stabilized and, therefore, readily detectable, even at high ion-trap temperatures. Thus, it is not surprising that the gain in sensitivity achieved by lowering the ion-trap temperature is smaller for alkadienes as compared to alkenes and saturated CHCs.

A decrease of the ion-trap temperature seems to be preferable over a decrease of the ion-source temperature as described by Doolittle et al. (1995), since the latter results in significant loss of sensitivity and a pronounced detector tailing, which hampers the identification of CHCs despite a greater intensity of diagnostic ions (data not shown). An increase of the ionization time of higher-mass scanning segments, as suggested by Doolittle et al. (1995) for internal ionization, gave no satisfactory results in our experiments with external ionization setup (data not shown). Although the abundance of higher mass ions increased considerably, the diagnostic value of these ions was low, as background ions from the sample or column-bleeding also were dramatically enhanced.

CI MS/MS of alkene adduct ions using ACN as a reagent gas allowed for the routine determination of the double bond position without the need for derivatization prior to analysis. This procedure previously has proven useful for the localization of double-bond positions in unsaturated hydrocarbons as well as fatty acid methyl esters (Moneti et al., 1997; Oldham and Svatos, 1999; Van Pelt and Brenna, 1999; Van Pelt et al., 1999; Michaud et al., 2002, 2003; Lawrence and Brenna, 2006). In comparison to DMDS derivatization followed by GC-MS, the ACN CI MS/MS method has the advantage of no shift in retention time of *in-situ* derivatized alkenes, which facilitates the assignment of the double-bond position. Additionally, high molecular weight alkenes and especially alkadienes, whose DMDS derivates have a high boiling-point and cannot be analyzed due to the temperature limit of many GC columns, can still be analyzed conveniently. Moreover, the CI MS/MS method can be conducted with comparatively low amounts of sample. It should be noted, however, that CI using ACN may significantly reduce the lifespan of the MS filament.

Our method allows for rapid, easy, and unambiguous identification of insect CHCs in one EI and one or two CI MS/MS runs, and it combines the advantages of quadrupole and ion-trap instruments. In summary, GC ion-trap mass spectrometry is a valuable tool for the identification of insect CHCs and offers alternative and complementary capabilities to quadrupole instruments.

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COMMENTARY

New Synthesis – Plant Defense Signaling: New Opportunities for Studying Chemical Diversity

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Higher plants have evolved multilayered defense signaling systems that are based on the generation and perception of small molecules. These signaling pathways provide warning of incoming danger, triggering not only local resistance responses but also long-distance signals that lead to the protection of the whole plant against subsequent attack. This induced resistance against pathogens and herbivores often involves the production of alkaloids, terpenes, phenolics, and other small molecules with toxic and deterrent properties. However, although there are thousands of known plant metabolites with direct defensive functions, relatively few signaling molecules have been identified. Among these, salicylate, jasmonate, and related metabolites are the most extensively studied, and likely have universal functions as components of pathways leading to the induction of both local and systemic plant defense responses.

Salicylate and jasmonate-dependent signaling pathways in plants are presumed to have their evolutionary origins in the production of metabolites with a direct defensive function. Salicin, a metabolic precursor of salicylate, has a protective function in plants such as willows, and lipid intermediates in jasmonate biosynthesis also have been implicated in plant defense. For plants under attack, the induction of a local defense response is a reliable indicator of pests or pathogens that have the potential to spread more widely. Small molecules that are phloem mobile or even volatile, e.g., methyl salicylate and methyl jasmonate, provide an efficient means for propagating a systemic signal. Thus, by eliciting additional defenses and turning on signaling pathways, secondary metabolites (SMs) can contribute to a signal amplification loop that prepares otherwise naïve plant tissue for imminent attack.

Plant defense signaling does not occur through linear pathways, but rather is a complex network with numerous examples of crosstalk and an ever increasing number of known signaling molecules. In particular, the accumulation of certain SMs not only provides direct protection against disease and herbivory, but also serves as a trigger for further plant defense responses. For instance, our own work shows that 4-methoxyindol-3-ylmethylglucosinolate in *Arabidopsis thaliana* not only protects against pathogens and herbivores, but also elicits callose production as a secondary defense response. Azelaic acid is a phloem-mobile metabolite that can prime salicylate-dependent plant responses (Jung et al., 2009) and, as suggested by its use as a human acne treatment, also has antimicrobial properties. Production of reactive oxygen species has long been implicated in plant resistance to disease and herbivory. More recently, hydrogen peroxide was reported as a key reactive oxygen signal in plant immune responses, acting downstream or independently of salicylate and jasmonate signaling pathways (Miller et al., 2009).

Just as the great diversity of plant SMs is required for defense against multiple threats, there is likely also natural selection for a diversity of defense signaling pathways. Species-specific pathways should be less susceptible to interference by herbivores and pathogens, either through the targeted degradation of plant-derived signals or by the insertion of interfering molecules, e.g., the jasmonate-isoleucine mimic coronatine, which promotes virulence of plant-pathogenic *Pseudomonas syringae* bacteria (Uppalapati et al., 2007). In this respect, defensive SMs may be particularly suitable as plant signaling molecules. These compounds exhibit great chemical diversity, generally are resistant to catabolism by non-specialist pathogens and herbivores, show increased local accumulation in response to infection or herbivory, and can spread through the plant vasculature to provide a systemic signal.

Given that the vast majority of plant SMs have been investigated primarily for their function as direct defenses, there are numerous opportunities for discovering as yet unidentified or incipient signaling pathways. New research tools, including metabolite profiling by mass spectrometry and analysis of gene expression by high-throughput sequencing, make it cost-effective to study global responses in non-model species. One approach would be an examination of plant responses to well-studied SMs, specifically to determine whether phloem and xylem-mobile toxins, e.g., nicotine, elicit additional systemic responses. New signaling molecules also may be discovered through systematic analysis of the countless unidentified plant metabolites that accumulate in response to pathogens and herbivores. Conducting such research with well-studied model species such as *A. thaliana*, rice, or tomato will make it possible to move beyond the identification of new signaling molecules to finding the genes and enzymes involved in their biosynthesis and response pathways.

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Production of Herbivore-Induced Plant Volatiles is Constrained Seasonally in The Field but Predation on Herbivores is not

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Abstract Natural enemies of herbivorous insects utilize numerous cues to locate and identify their prey. One class of such cues is volatile organic compounds (VOCs) often produced by plants after attack by herbivores (hereafter herbivore-induced plant volatiles or HIPVs). Under simplified laboratory conditions, natural enemies often make clear choices between different HIPV blends, but such clear choices may be more difficult in more complex field environments. We studied the impact of VOC production by the undomesticated species, *Datura wrightii* on predation of eggs and larvae of *Lema daturaphila* by the omnivore, *Geocoris pallens* in the field. HIPV production in *D. wrightii* is developmentally and seasonally constrained to the early stages of plant growth even though *L. daturaphila* and *G. pallens* inhabit plants throughout the plant's growing season. We, therefore, asked if predation of *L. daturaphila* by *G. pallens* might be similarly constrained seasonally. Higher levels of VOC production were associated with higher levels of predation throughout the growing season, and the greater quantities of VOC production in May caused greater increases in predation than did VOC production later in the season (June–September). However, predation in the absence of VOC production ranged from 60–70% in June–September compared to only 14% in May, probably because plants were already colonized by predators later in the season. High levels of VOCs in response to herbivore damage by *D. wrightii* therefore may aid in the discovery of herbivore-damaged plants early in the season but the seasonal decline in VOC production does not limit predation of *L. daturaphila* by *G. pallens* later in the season.

Key Words Induced defense · Ontogenetic variation · Indirect defense · VOCs · HIPVs · Tritrophic interactions · Predation · *Datura wrightii* · *Lema daturaphila* · *Geocoris pallens*

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Introduction

Predators and parasitoids of plant-feeding insects utilize a range of visual and chemical cues to guide them through the stepwise process of first locating a damaged plant, then locating a potential host on that plant, and ultimately assessing the quality of a host individual for utilization (Vinson, 1976). Among the chemical cues are the inducible plant odors that facilitate the discovery by natural enemies of the habitats of potential prey species (Vinson, 1976, 1981; Nordlund et al., 1988). Many of the plant odors are inducible and only released by plants after they have been damaged by herbivores (Elzen et al., 1983; Dicke and Sabelis, 1988; Turlings et al., 1991; Tumlinson et al., 1992; Vet and Dicke, 1992). These compounds are termed herbivore-induced plant volatiles (HIPVs) and are a subset of the more general class of volatile organic compounds (VOCs) emitted by plants either constitutively or in response to other plant stresses. Inducible VOCs may serve as relatively long distance cues to signify that a plant may be infested with potential hosts, although "long distance" rarely is defined (Puente et al., 2008).

Most studies on the role of HIPVs on the behavior of natural enemies have been performed under controlled laboratory conditions (Hunter, 2002; Turlings and Wäckers, 2004; Allison and Hare, 2009). The laboratory environment, however, greatly simplifies the choices that natural enemies need to make, and it eliminates the effect of biotic and abiotic factors that may complicate the behavioral choices that natural enemies would make under more natural field conditions. Such factors would include, among other things, variation in light intensity, variation in the time since damage occurred, damage by non-host herbivores, and the potentially interfering volatiles from other species of plants (Takabayashi et al., 1994; Gouinguene and Turlings, 2002; Gols et al., 2005; Bukovinszky et al., 2007). These differences between the field and laboratory environments therefore raise questions as to whether the behavioral responses observed under laboratory conditions commonly occur under the more complex field environments. Additionally, most field research is performed on domesticated plant species far removed from their natural habitats and grown under simplified conditions. Such conditions may not represent the more complex environments in which any interactions among plants, their herbivores, and the natural enemies of those herbivores might have evolved. Finally, the available studies performed in the field often assess the attraction of natural enemies to plants only at selected times during plant development (De Moraes et al., 1998; Thaler, 1999; Kessler and Baldwin, 2001; Halitschke et al., 2008), although ontogenetic variation in the ability of some plant species to emit HIPVs has been demonstrated (Köllner et al., 2004; Rostas and Eggert, 2008; Hare, 2010). All of these factors may add complications to the use of HIPVs by natural enemies under natural conditions. The rarity of studies on tritrophic interactions on undomesticated plant species in their natural environments is a major limitation to confirm, if not challenge, the results from the laboratory to develop a thorough and accurate understanding of plant-mediated interactions between herbivores and their natural enemies (Turlings and Benrey, 1998; Dicke and van Loon, 2000; van der Meijden and Klinkhamer, 2000; Hare, 2002; Janssen et al., 2002; Turlings and Wäckers, 2004).

One system in which the ecological significance of VOC production can be investigated under natural conditions involves the undomesticated plant species *Datura wrightii* (Solanaceae) and its native herbivores and natural enemies in its native environment. Previous research showed that, in the laboratory, young *D. wrightii* plants could be induced to produce blends of VOCs either when fed upon by the specialist chrysomelid beetle, *Lema daturaphila*, or when treated with the plant hormone methyl jasmonate (MeJA) (Hare, 2007). Different genetic lines of *D. wrightii* differed in both the quantities of inducible VOCs as well as the composition of the VOC blends, and the quantitative differences were heritable (Hare, 2007).

Unlike most of the rapidly-growing annual species in which HIPV production has been studied, *D. wrightii* has a long growing season in the sage scrub habitats of inland southern California. Seeds germinate in March or April after winter rains, but flowering usually is not initiated until June, after which plants continue to grow while also producing flowers and seed capsules through November. HIPV production in *D. wrightii* is confined largely to the early part of the plant's growing season when plants are growing vegetatively, and it declines as plants begin to flower and produce fruit (Hare, 2010). Similar levels of herbivory by *L. daturaphila* resulted in the production of nearly 25-times more HIPVs when inflicted on plants in April than when inflicted in August or September. Ontogenetic constraints on HIPV production were further demonstrated by showing that HIPV production could be partially restored simply by cutting plants back to ground level in mid-summer and allowing them to re-sprout and re-grow (Hare, 2010). Interestingly, despite the clear seasonal variation in HIPV production, the most important herbivores of *D. wrightii, L. daturaphila* and the mirid bug, *Tupiocoris notatus*, and their shared natural enemy, the omnivorous Western big-eyed bug, *Geocoris pallens*, (Heteroptera: Lygaeidae) inhabited plants throughout the plant's growing season (Hare, 2010).

Geocoris pallens and/or its more tractable congener, *G. punctipes,* respond positively to several individual VOC components, including linalool, α-bergamotene, 2-hexenal, indole, and methyl salicylate (MeSA) under field conditions depending upon the precise details of the experimental design (Kessler and Baldwin, 2001; James, 2003, 2005; Halitschke et al., 2008). In the wild tobacco species, *Nicotiana attenuata, G. pallens* was attracted to plants damaged either by *T. notatus* or *Manduca* spp. such that *G. pallens* inflicted 4.6-fold higher mortality of *Manduca* eggs experimentally placed on *T. notatus*—damaged plants in the field (Kessler and Baldwin, 2004). In addition to possibly using plant chemical cues to locate potential prey, *Geocoris* spp. also utilize their acute sense of vision (Eubanks and Denno, 2000).

Here, we investigated the impact of seasonal and genetic variation of VOC production in *D. wrightii* on predation of eggs and larvae of *L. daturaphila* by *G. pallens* in the greenhouse and field. Two trichome phenotypes occur in natural populations of *D. wrightii* in southern California, with some individuals producing leaves with short, non-glandular trichomes, and others producing leaves with longer glandular trichomes (van Dam et al., 1999). Prior laboratory studies showed *G. pallens* to be a more effective predator on plants with nonglandular trichomes (Gassmann

and Hare, 2005). We, therefore, investigated the role of variation in trichome morphology on predation by *G. pallens*. Our specific goals were to determine 1) if predation by *G. pallens* was greater on induced than on uninduced plants, 2) if the seasonal decline in HIPV production caused any seasonal decline in predation rates, 3) if genetic lines of plants that produced greater quantities of HIPVs and/or different HIPV blends benefitted from greater predation of *L. daturaphila*, and 4) if predation rates were lower on plants producing glandular trichomes than on plants producing nonglandular trichomes, as previous laboratory studies would predict. Our experimental designs also included the opportunity to test for all two- and three-way interactions between trichome morphology, genetic line, and plant developmental stage on HIPV production and predation by *G. pallens*.

Methods and Materials

The Study System Aspects of the ecology and genetics of the trichome dimorphism have been described elsewhere (van Dam and Hare, 1998b; van Dam et al., 1999). Leaves of the 'velvety' phenotype are densely covered by short, non-glandular trichomes and feel velvety to the touch, whereas leaves of the 'sticky' phenotype are less densely covered by glandular trichomes that secrete esters of glucose and aliphatic acids, and these leaves feel sticky to the touch. The trichome phenotype is governed by a single locus and is inherited in a Mendelian fashion; the allele for the sticky phenotype is dominant over that of the velvety phenotype (van Dam et al., 1999). Under natural conditions, *D. wrightii* is embedded in a community of plants, each with their own communities of herbivores and natural enemies. Plants occur in patches that persist for several years (Hare and Elle, 2004). Individual plants within patches quickly become colonized by overwintering adults of *L. daturaphila*, *T. notatus*, and other herbivores in the early spring, then suffer chronic herbivory throughout the growing season. The herbivore community attacking *D. wrightii* is relatively simple, being comprised of some five to ten species, depending upon season and location (Elle and Hare, 2000; Hare and Elle, 2002). The most abundant herbivore species overall is *L. daturaphila*, a folivore whose adult and larval stages impose chronic, season-long herbivory on both trichome phenotypes. A number of generalist natural enemies are associated with the herbivores of *D. wrightii*, but the most abundant is the omnivore, *G. pallens* (Gassmann and Hare, 2005). We have confirmed that *G. pallens* behaves poorly in several different laboratory olfactometers (see also Halitschke et al., 2008) but it does behave more naturally in larger-scale greenhouse experiments to be described below.

The plant lines used in these experiments were fifth-generation backcross progeny of heterozygous sticky progeny backcrossed to their original velvety pollen parent. The expected ratio of adult heterozygous sticky to homozygous recessive velvety sibs within each of the lines derived from each of eight original pollen donors is 1:1, and sibs within families are expected to be at least 98.4% similar after five generations of backcrossing. Seeds were germinated in the winter of 2009–2010 in a greenhouse equipped with high-pressure sodium lamps providing supplemental illumination for 16 h/day so that mid-day light intensities averaged 1,250±39 μmol m^{-2} s^{-1} PAR illumination (N=5 readings) at plant height. Greenhouse temperatures ranged between 15° and 35°C. Plants were maintained under these conditions for about 3 mo until trichome differentiation was complete (van Dam et al., 1999).

Greenhouse Experiments For the greenhouse experiments, fifth-generation backcross progeny from the highly inducible MVV6 and MVV8 lines were used (Hare, 2007). Survival of newly-hatched first-instar *L. daturaphila* was monitored on induced and uninduced sticky or velvety plants in the presence or absence of *G. pallens* in two separate experiments. In the first experiment, plants were induced by feeding by adult *L. daturaphila* on two young but fully expanded leaves of test plants for 48 h prior to the beginning of the experiment. In the second experiment, plants were induced by spraying the plant with 8 mM MeJA in 0.1% Tween 20® in water just to runoff. These treatments routinely induce similarly high levels of HIPV production in young *D. wrightii* (Hare, 2007). Induction treatments were initiated 48 h prior to the beginning of the predation experiment because maximum HIPV production usually occurs within 2–3 d after induction (Hare, 2007). Five newly-hatched first-instars of *L. daturaphila* were placed on the lower surface of each of the two leaves damaged by *L. daturaphila*, or leaves of similar age for uninduced plants and plants induced by MeJA. The plants were placed near the corners of a cage 1 m^3 in size. A three-piece hardboard floor with overlapping slots for the plant stems was placed on top of the pots and around the plants so that the stems emerged from a uniform surface. A quantity of shredded paper toweling was placed in the center of the cage to provide cover. Groups of 20 *G. pallens* were collected from the field, starved for 48 h, then released into the shredded paper toweling in the cages. Each group of treatments was repeated in 3–5 cages per wk, and the whole experiment was replicated over several weeks. One 'control' cage, containing plants only with *L. daturaphila* larvae and no predators, also was established within each weekly trial. After releasing the group of 20 adult *G. pallens,* survival of *L. daturaphila* larvae was assessed 72 h

later. By this time, surviving larvae had reached the 2nd or early 3rd instars but were still susceptible to predation by *G. pallens* (*pers. obs.*). In sum, there were 4 control and 8 predation replicates in the experiments utilizing MeJA as the inducer and 3 control and 11 predation replicates in the experiments utilizing damage by *L. daturaphila* adults as the inducer.

Field Plot Layout Plants used in this experiment included fifth-generation backcrosses of the UCV9, UCV8, OFV8, and OFV6 lines described previously (Hare, 2007). In the greenhouse, the UCV9 line produced relatively low levels of VOCs after induction, whereas the OFV6 line produced relatively high levels, and the other two lines produced intermediate levels (Hare, 2007, Fig. 3). Seeds were planted in a greenhouse and allowed to germinate and differentiate according to adult trichome type, then transplanted at the 8- to 10-leaf stage to the field on 21 April, 2010. Plants were transplanted adjacent to a 3-yr-old plot of *D. wrightii* (ca. 46×40 m) that was maintained in a 'naturalized' condition with no weed control and no irrigation after normal winter rains. Both herbivorous insects and natural enemy populations had become established in this field in previous years.

Experimental plants were planted in rows 2.7 m apart with plants spaced 2.7 m apart within rows and 0.8 m between rows. Plants were staggered between rows to maximize separation between plants in adjacent rows. Each row contained 16 plants with a pair of sticky and velvety plants from each of the four lines, and sticky and velvety plants were planted alternately. The closest row to the naturalized field was 1.8 m away. The new plot was treated with a pre-emergence herbicide (Trifluralin at the rate of 668 ml/ha) for weed control prior to transplanting. The plot was furrow-irrigated prior to transplanting for 24 h, then for 8 h, 3 d after transplanting, and weekly for 8 h for 3 wk thereafter. The plants were not irrigated after 17 May, 2010, and no rainfall occurred during the experiment. The absence of any rainfall during the spring and summer is characteristic of the sage scrub habitats in which *D. wrightii* commonly occurs.

VOC Production and Predation in the Field Experiments were carried out five times during the growing season during the weeks of 10 May, 10 June, 15 July, 5 August, and 2 September using five sets of adjacent pairs of rows providing a maximum of 32 plants per experiment. Plants were protected from natural attack by *L. daturaphila* prior to utilization in experiments with carefully-timed applications of acephate (Orthene 97, Valent Chemical Co., Walnut Creek, CA, USA 1.2 g/l) in water, and applications were stopped at least 1 wk prior to the initiation of each experiment. Prior to each experiment, plants were tested against *L. daturaphila* to determine that they were no longer toxic. This was verified by caging adult *L. daturaphila* on a similarly-treated set of plants and noting insect survival 2 d later.

To start each experiment, two branches of similar age and leaf production on each of 16 plants were infested with a pair each of field-collected adult *L. daturaphila* contained in a small cage enclosing a terminal of leaves. Adults were allowed to feed for 48 h to induce VOC production. The remaining 16 plants remained uninfested. After the 48 h induction period, the VOC production from damaged and undamaged plants was determined using procedures described previously (Hare, 2010). Briefly, aeration chambers were made from polyester cooking bags (unprinted 45× 55 cm bags, Terinex, Bedford, U.K.) and placed over the terminal leaves of a branch. Each chamber enclosed 4–6 full-sized leaves and a similar number of developing leaves. For the damaged plants, adult insects were removed before aerating branches with damaged leaves. VOCs were adsorbed on traps made of 4.6-mm O.D. glass tubing and filled with 25 mg of Super-Q (Alltech, State College, PA, USA). Air was introduced into the bag through PTFE tubing (4.6-mm O.D.) and was withdrawn from the bags through the traps through PVC tubing (4.6-mm I.D.) downstream of the traps in a "push-pull" scheme using two 12-V portable air pumps (Model # MOA- P125-JH, Gast Manufacturing, Benton Harbor, MI, USA). A 12-V deep-cycle marine battery powered both pumps. A manifold with 16 pairs of flow meters (Aalborg Instruments, Orangeburg, NY, USA) was constructed so that inlet and exhaust flow to and from the sampling bags could be precisely and independently regulated at 1.0 l/min for each plant. Inlet air was drawn through a filter of activated charcoal placed between the pump and the manifold. Preliminary studies showed that the charcoal filter was sufficient to remove all ambient VOCs. Plants were aerated in groups of up to 16 for 90 min and two groups of plants were aerated on each sampling day between the hours of 08.30 h and 10.00 h for the first group and between 11:00 h and 12.30 h for the second group. Data on air temperature, relative humidity, and light intensity, recorded hourly on each sampling day from 09.00 h until 13.00 h were obtained from records from the California Irrigation Management Information System (CIMIS) weather station located no more than 90 m from the experimental plot (Table 1). At the termination of the aeration, traps were wrapped in aluminum foil, labeled, returned to the laboratory, and stored at −20 ° C for extraction later that day. The twist-tie used to seal the bags was reattached to the branch at its original location to identify the branch and aerated leaves.

Volatiles were eluted from the traps with 150 µl of CH_2Cl_2 containing 4 ng/µl of 1-bromoheptane as an internal standard into autosampler vials with 250 µl glass

Table 1 Mean (± standard error) temperature, relative humidity, and light intensity during the aeration periods on each sampling date in 2010. Means and standard errors were calculated over the 5-h aeration period (09.00–13.00 h) on each date

Date	Temperature (°C)	Relative humidity (%)	Light intensity ($w \cdot m^{-2}$)
10 May	16.5±0.7	48±3	648±53
10 June	18.6±1.2	68±6	540±148
15 July	34.4±1.1	27±2	701±54
5 August	23.6±1.9	55±8	719±60
2 September	32.1±1.9	26±5	700±59

inserts and sealed with crimp caps and PTFE-lined rubber septa. Samples were analyzed by gas-liquid chromatography largely as described in Hare (2010). To confirm identifications, selected samples were analyzed with a Hewlett- Packard 6890 gas chromatograph coupled to a Hewlett- Packard 5973 mass selective detector, and the mass spectra and retention times of compounds were matched with those of authentic standards as described in Hare (2007). Peak quantification, in units of $ng \cdot g^{-1}$ leaf (dry wt.)$\cdot h^{-1}$, was made using Agilent ChemStation® software based on comparison of their peak height with that of the internal standard and leaf dry weights at the end of the experiment.

Prior to aeration, clusters of ten *L. daturaphila* eggs were prepared by dividing natural egg clusters collected from field-grown *D. wrightii* outside of the experiment into groups of ten and placing them on a disc 1 cm diam punched from a *D. wrightii* leaf collected from plants not in the experimental plot. The natural adhesive on these eggs was sufficient to affix the eggs to the leaf disk. After plants were aerated, two to four leaf disks, depending upon plant size, were glued to the lower surface of the aerated leaves with a synthetic resin adhesive (UHU Adhesives, Saunders Mfg. Co., Readfield, ME, USA). Half of the disks were enclosed in 'closed' clip cages constructed from spring hair clips and 2.5-cm diam plastic vials glued to rubber washers (1.8-cm I.D., 2.5-cm O.D.) The caps of the vials were bored out and covered with brass screen to provide ventilation. The other half of the egg masses were enclosed in 'open' cages constructed from hair clips and rubber washers only. The 'open' cages provided free access to predators and controlled for the possibility of subsequent induction of VOCs from mechanical wounding of the leaves caused by the cages themselves. In many cases, especially in the later trials, we noted that experimental plants already were colonized by *G. pallens* even before placing experimental eggs on plants.

After 4 d in the field, branches with cages were removed and returned to the laboratory. The number of hatched eggs was determined, and disks with unhatched eggs were removed from their leaves and assessed for damage under a microscope. Aerated leaves were then dried to a constant weight (80°C, 48 h) then weighed.

Eggs attacked by predators were easily recognized by their shriveled appearance. Any unhatched eggs were monitored for an additional 5 d. Because first-instars feed in groups after hatching and do not disperse, it was relatively simple to reconcile the number of larvae with the number of hatched, unhatched and shriveled eggs in both closed and open cages. Although mortality in the closed cages was quite low (see Results), it did differ among dates, lines, and the date by line interaction. Thus, the mortality due to predation must take into account these sources of mortality in the absence of predation. Accordingly, the mean mortality in the closed cages was calculated on a per-plant basis and was subtracted from the observed mortality in each of the open cages for each plant to estimate mortality due to predation. Values for predation could take on non-integer values if mortality in the two closed cages per plant were not equal, and on two occasions, calculated predation was −1 when all ten eggs in the open cages completely escaped predation and only nine eggs hatched in the closed cages. Although the initial design encompassed 160 plants, data were collected from 110 due to accidental loss of samples (leaky fittings, punctured bags), and limitations on the number of experimental adults and larvae for the experiments. Most plants used after the May aerations were large enough for two pairs of cages so the final sample size for the predation experiments was 208.

Statistical Analysis For the greenhouse experiments, larval survival was converted to a percentage then analyzed by two-factor restricted maximum likelihood (REML) ANOVA using PROC MIXED of SAS (SAS Institute 2008) separately for larvae exposed to and protected from predators. Fixed effects included trichome phenotype, induction status, and their interaction. The arcsine square root transformation was applied to ensure normality of errors.

For the field experiments, the data on predation were analyzed by a REML ANOVA using PROC MIXED. The full ANOVA model on predation included 1) the fixed effects of damage status (damaged or undamaged), genetic line, and trichome type, 2) all two- and three-way interactions of the fixed effects, and 3) replicate plants

within date by line by trichome type combinations and the 'row within date', a surrogate for the distance of plants from the source of the predator population, as random effects.

Because the effect of induction on VOC production was expected to vary over time (Hare, 2010), we also examined VOC production directly on predation over the full season by analysis of covariance (ANCOVA) by replacing the categorical variable of damage status with the continuous variable of observed VOC production. All interactions, including the potential interaction between VOC production and all fixed effects, were included in the full model. A significant interaction between VOC production and date, for example, would indicate that the linear relationships between predation and VOC production varied over time. Where appropriate, two-way interactions were decomposed using the SLICE option of PROC MIXED. For all VOC analyses, the $\log_{10}$ (x+1) transformation was applied prior to analysis to ensure normality of errors; the x+1 transformation was required because some VOC components were not detectable, especially late in the season. Predation was converted to a percentage and required no transformation.

To explore the potential importance of variation in the composition of VOC blends on predation, we also performed an exploratory Principal Components Analysis (PCA) based upon the covariance matrix of samples from all 110 plants to calculate a smaller number of uncorrelated components that were linear combinations of the original variables and to identify particular components contributing most to the patterns of variation in VOC production. The factor loadings show how much each of the original variables contributes to each new component, and factor scores for the new components were calculated for each original observation. An ANCOVA on these factor scores (PCA-ANCOVA) was used to determine the influence of the principal components on predation after accounting for the other sources of variation of sampling date, genetic line, trichome phenotype, and their interactions.

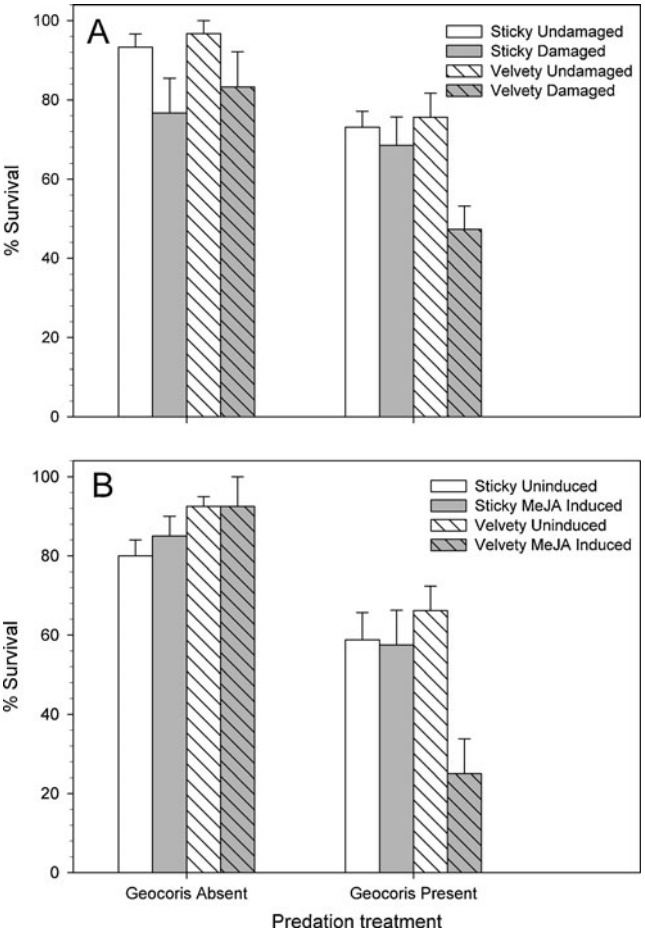


Fig. 1 Mean (+ standard error) percent survival of *Lema daturaphila* larvae 72 h after being placed on induced or uninduced plants in cages in the presence or absence of *Geocoris pallens.* Plot A: Plants induced by damage from feeding by adult *L. daturaphila* for 48 h prior to the beginning of the experiment. Plot B: Plants induced with 8 mM MeJA in 0.1% Tween 48 h prior to the beginning of the experiment. White bars: undamaged plants; shaded bars, insect-damaged plants; open bars, sticky trichome phenotype; hatched bars, velvety trichome phenotype

Results

Greenhouse Experiments For the plants induced by feeding damage by adult *L. daturaphila,* survival of larvae of *L. daturaphila* in the absence of *G. pallens* averaged 87.5% and did not differ significantly due to trichome phenotype ($F_{1,\ 8}$=0.95, P=0.36), induction status ($F_{1,\ 8}$=4.06 P=0.08), nor due to their interaction ($F_{1,\ 8}$=0.02, P=0.89, Fig. 1A). In the presence of *G. pallens,* overall survival was reduced to 66% but differed due to induction status ($F_{1,\ 44}$=6.61, P=0.01) and also due the interaction between induction status and trichome type ($F_{1,\ 44}$=4.06, P=0.05); predation was greatest on the induced velvety phenotype (Fig. 1A). The main effect of trichome type was not statistically significant, however ($F_{1,\ 44}$=1.53, P=0.22). The initial analyses included the replicate week as a random effect but this was deleted from the final model because it was never statistically significant.

For the experiment with MeJA as the inducer, survival in the absence of predators also averaged 87.5% but was lower on sticky than on velvety plants ($F_{1,\ 12}$=6.07, P=0.03, Fig. 1B). Survival did not differ due to induction status or the trichome phenotype by induction interaction (both $P \geq 0.38$). In the presence of predators, however, survival dropped to an average of 51.8% but differed due to trichome phenotype ($F_{1,\ 28}$=6.25, P=0.019) and due to the trichome by induction status interaction ($F_{1,\ 28}$=4.82, P=

0.037). In both experiments, the significant trichome type by induction status demonstrates that predation by *G. pallens* was greatest on induced velvety plants.

Total VOC Production in the Field When including damage status (i.e., damaged or undamaged) as a fixed effect, total VOC production varied only over time ($F_{4,\ 66}$=52.96, P< 0.001). The effect of damage was not statistically significant overall ($F_{1,\ 66}$=3.19, P=0.07), but VOC production was higher in insect-damaged plants than in undamaged plants in May ($F_{1,\ 14}$=5.12, P=0.04) and in June ($F_{1,\ 14}$= 4.58, P=0.05, Fig. 2). Overall VOC production was substantially lower in July–September (Fig. 2), and not different between damaged and undamaged plants (all $P\geq$ 0.15, Fig. 2). No other main effect (genetic line, trichome phenotype) or any of the two-, three-, or four-way interactions were statistically significant.

Predation: Preliminary Analyses Monthly mean percent (± s.e.) egg hatch in closed cages ranged from a low of 80± 2% in September to a high of 92±2% in June when pooled over lines and trichome types (data not shown). These high values of survival show that mortality due to handling or residual insecticide activity on the plants was minimal. The observed larval mortality differed among dates ($F_{4,\ 158}$= 18.47, P=0.004), lines ($F_{3,\ 158}$=2.93, P=0.035), and the date by line interaction ($F_{12,\ 158}$=2.05, P=0.023). Natural mortality in the closed cages, therefore, was used to calculate additional mortality due to predation in the open cages on a per-plant basis (Fig. 3).

When analyzed by ANOVA that included damage status as a fixed effect (damaged vs. undamaged), predation varied significantly over time ($F_{4,\ 171}$=19.56, P<0.001) and due to the damage status by date interaction ($F_{4,\ 171}$=3.07, P=0.018), the date by genetic line interaction ($F_{12,\ 171}$=2.55, P=0.004), and the genetic line by trichome type interaction ($F_{3,\ 171}$=3.29, P=0.02). Nonsignificant interactions were deleted from the final model. The random factors of row within date, and plant within date by line by trichome type combinations were also dropped from the final model because they did not account for a significant portion of the total variation (P=1.00 for rows within dates and P=0.75 for replicate plants within treatment combinations). The significant two way interaction between damage status and date resulted from predation being higher on damaged than on undamaged plants in May, July, and August, but not in June or September. The interaction between genetic line and trichome was because predation was higher on velvety than sticky plants overall for all lines except OFV8, and the date by genetic line interaction was because predation on the different lines varied in a complex way over time. The seasonally variable effect of damage status may not be surprising, given the substantial seasonal variation in VOC production of damaged and undamaged plants (Fig. 2). More meaningful results were obtained by replacing the fixed effect of damage status by the continuous variable of VOC production for each damaged or undamaged plant.

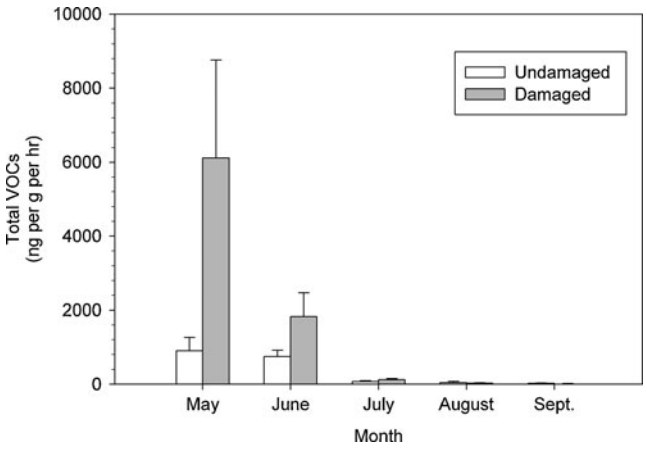


Fig. 2 Mean (+ standard error) total VOC production (ng·g^{-1} leaf (dry wt.)·h^{-1}) of undamaged plants or plants damaged by 48 h feeding by adult *Lema daturaphila* in the field for each monthly experiment. White bars: undamaged plants; shaded bars, insect-damaged plants

Predation and Relationship to Total VOC Production In the final model for the analysis of predation in the field, predation increased with increasing VOC production (slope=9.91±2.14, $F_{1,\ 179}$=21.48, P<0.001). The adjusted treatment means for percent predation (i.e., the expected predation in the absence of VOC production) also varied among sampling dates ($F_{4,\ 179}$=25.80, P<0.001, Fig. 3). The lowest value was 14.5±5.3 % in May, and the values for the other 4 months ranged from a low of 60.0±4.1% in June to a high of 70.4±2.6% in July. Nonsignificant interactions and the two nonsignificant random factors, replicate plants within date by line by trichome type combinations and row within date were dropped from the final model. There were no significant interactions between VOC production and any of the fixed effects or their interactions (all $P\geq$0.33), indicating nonsignificant differences in the slopes of the regression lines relating predation to VOC production across all treatment combinations; this justifies using VOC production as a simple covariate. After adjusting for the variation in VOC production, then neither genetic line nor trichome phenotype were statistically significant as main effects ($P\geq$0.55), but the date by trichome phenotype interaction and the line by trichome phenotype interactions both were statistically significant ($F_{4,\ 179}$=3.46, P=0.009 and $F_{3,\ 179}$=4.41, P=0.005, respectively). The date by trichome phenotype interaction was caused by predation differing between trichome phenotypes only in the month of August ($F_{1,\ 179}$= 12.72, P<0.001) and was higher on velvety plants. The line

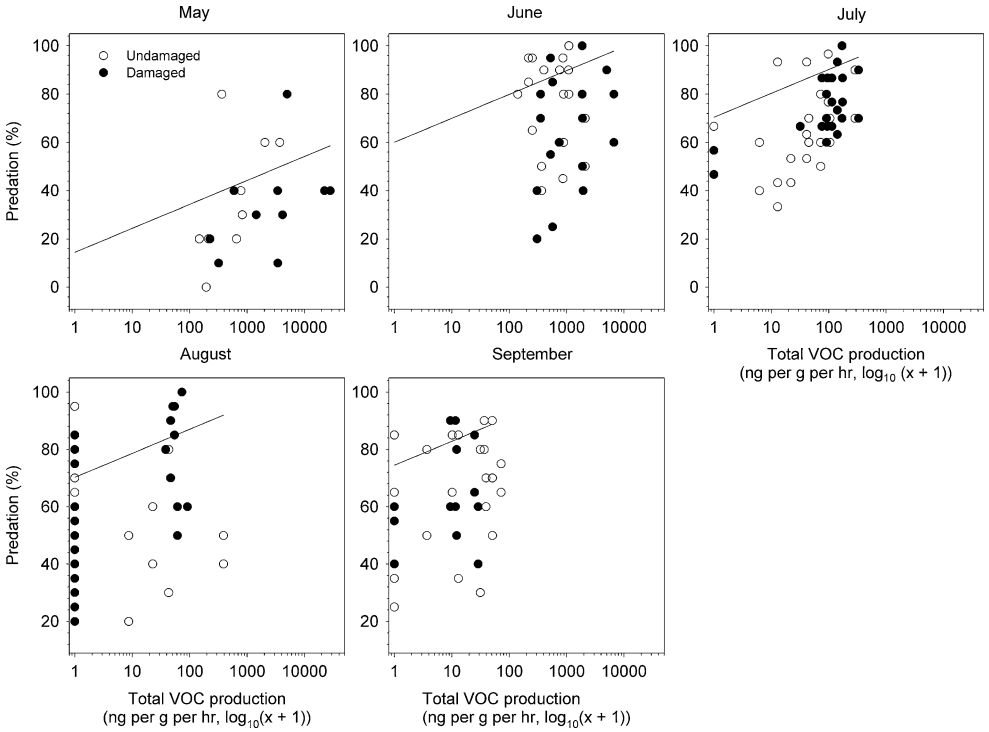


Fig. 3 Predation (%) and VOC production (ng·g^{-1} leaf (dry wt.)·h^{-1} $\log_{10}$ (x+1) transformation) for insect-damaged plants (filled circles) and undamaged plants (open circles) for each monthly experiment. Response lines were drawn using the common slope relating VOC production to predation from ANCOVA over the range from the *y*-intercept, i.e., the expected predation in the absence of VOC production, to the maximum VOC production observed for each monthly experiment. Data were pooled over trichome phenotype and genetic lines because these factors were not statistically significant in ANCOVA

by trichome phenotype interaction was caused by predation differing between trichome phenotypes only for the UCV9 line ($F_{1,\ 179}$=5.59, P=0.019), and was higher on sticky plants.

In summary, although predation increased with increasing VOC production over all sampling dates, the baseline predation of *L. daturaphila* by *G. pallens* in the absence of VOCs was ca. 4–5 times higher in June through September than it was in May (Fig. 3). The data for the month of May had two plants with extremely high VOC production after damage, and it was possible that these results might have been unduly dependent upon the data from these two plants. Accordingly, the data were reanalyzed after deleting these two extreme plants, and the pattern of statistically significant differences was unchanged. The absence of an overall effect of trichome phenotype and the inconsistent effects of interactions including trichome phenotype may suggest that the greenhouse experiments overestimated the effect of the trichome dimorphism on predation by *G. pallens* in the field.

Predation and Relationship to the Composition of VOC Blends Seven principal components were extracted. PC 1 accounted for more than 60% of the total variation, and the remaining six accounted for additional, independent proportions of the total variance ranging from 9.5 to 2.4% (Table 2). All individual VOC components except one loaded positively on PC 1, indicating that this component is a "general production" component. The individual compounds had positive or negative loadings in the other PCs, indicating relative differences in the composition of the blend. PC 2, for example, had positive loadings for most compounds but high negative loadings for (*E*)-β-caryophyllene and (*Z*)-3 hexenyl acetate. This component contrasts plants that produced high levels of these two compounds with plants that produced relatively high levels of all others. PC 3 independently separates blends relatively rich in (*E*)-β-ocimene from those relatively rich in (*E*)-β-caryophyllene, but the interpretation of the other PCs are less obvious (Table 2). Although there was statistically significant variation in both the overall quantity of the blend (PC 1) and different aspects of the composition of the blends (PCs 2–7, Table 3) only the variation in PC 1 was significantly related to predation; predation increased linearly with PC 1 ("general production") but was not statistically associated with the variation in the composition of the blends (Table 4). The variation in the composition of the blends among genetic lines of plants or over time, therefore, will not be presented in detail.

Discussion

The greenhouse experiments showed significantly increased predation of *L. daturaphila* larvae by *G. pallens* on herbivore- or MeJA-induced plants, especially on the velvety trichome phenotype. The results from the field experiments were more complex. VOC production was positively related to predation overall, but the overall

Table 2 Factor loadings, eigenvalues, and proportion of total variance from the Principal Components Analysis on VOC production

Compound	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
Trans-2-hexenal	−0.003	−0.003	0.025	0.009	−0.012	−0.010	−0.019
Trans-2-hexen-1-ol	0.016	0.031	0.011	0.027	0.066	0.156	0.026
α-Pinene	0.044	−0.006	0.004	0.016	0.065	0.313	0.030
Hex Ac[a]	0.425	−0.256	0.062	0.833	0.102	−0.133	−0.100
Limonene	0.054	0.082	0.096	0.100	0.359	0.581	−0.129
(*E*)-β-Ocimene	0.532	−0.040	0.723	−0.305	−0.243	−0.063	−0.068
Linalool	0.330	0.837	−0.247	0.091	−0.087	−0.165	−0.163
DMNT[a]	0.037	0.043	0.034	0.020	−0.008	0.232	0.222
MeSA[a]	0.045	0.109	−0.012	0.053	−0.261	0.127	−0.047
(*E*)-β-Caryophyllene	0.589	−0.358	−0.592	−0.331	0.058	−0.020	0.159
α-Bergamotene	0.047	0.019	0.038	−0.015	0.240	0.308	−0.016
Geranyl Acetone	0.092	0.221	0.086	0.142	−0.036	0.101	0.879
α-Humulene	0.200	0.051	−0.105	−0.080	−0.251	0.510	−0.225
β-Selinene	0.019	0.044	0.002	−0.077	0.020	−0.057	0.059
Nerolidol	0.014	−0.020	0.046	0.012	−0.007	0.086	0.119
TMTT[a]	0.017	0.098	−0.041	−0.025	0.211	−0.012	−0.148
(*E, E*)-α-Farnesene	0.146	0.138	0.164	−0.218	0.739	−0.212	0.017
Eigenvalues	3.84	0.60	0.49	0.41	0.26	0.20	0.15
Proportion	60.2	9.5	7.7	6.5	4.0	3.2	2.4

[a] Hex Ac: (*Z*)-3-hexenyl acetate; DMNT: (*E*)-4,8,dimethyl-1,3,7 nonatriene; MeSA: methyl salicylate; TMTT: (*E*)-4-8,12 trimethyl-1,3,7,11 tridecatetraene

increase in predation due to VOC production was less during the summer months than in the spring due to the overall seasonal decline in VOC production in response to damage. The differences in predation between damaged and undamaged plants varied over the season, and the apparent benefits of VOC production declined over the season. Simultaneously, overall levels of predation increased over the season even in the absence of VOC production, and we speculate that the omnivorous *G. pallens* congregated on *D. wrightii* and other perennials during the summer as the spring-growing annual plant species senesced and died. The utility of VOCs to facilitate predation of eggs and young larvae *of L. daturaphila* by *G. pallens* in the field, therefore, varied seasonally but correlated reasonably well with the time period when plants produced maximum levels of VOCs.

VOC production was not an explicit indicator of damage by herbivores. Although VOC production was significantly higher in damaged than undamaged plants in May and June, mean VOC production by undamaged plants during these months exceeded that for damaged plants later in the season (Fig. 2). Depending upon the particular plant species and experimental conditions, the production of plant volatile compounds can be elicited by factors other than herbivory, including simple mechanical wounding (Mithofer et al., 2005), exposure to UV light, (Johnson et al., 1999), and ozone (Vuorinen et al., 2004). Although the role of these potential abiotic factors in inducing volatile production in young *Datura* has not been investigated, their potential to contribute to the background production of volatile organic compounds under field conditions cannot be ignored.

Table 3 Results of REML ANOVA on factor scores from Principal Components Analysis. For each effect and their interactions, the numerator degrees of freedom, denominator degrees of freedom, and the *F*-statistic are shown for PC1 through PC 7. Statistically significant *F*-statistics ($P \leq 0.05$) are shown in bold

Effect	Numerator df	Denominator df	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
Sampling Date	4	70	**70.82**	**5.11**	1.59	1.72	0.77	**4.96**	0.66
Genetic Line	3	70	1.03	1.70	**3.11**	**3.27**	0.24	**3.76**	1.10
Date by Line	12	70	**2.26**	0.61	**2.59**	0.78	0.23	1.41	0.66
Trichome Phenotype	1	70	0.00	2.07	0.05	0.03	0.15	0.19	2.04
Date by Trichome Phenotype	4	70	0.43	1.65	1.68	0.37	0.34	0.26	0.23
Line by Trichome Phenotype	3	70	0.94	0.24	0.45	0.45	0.26	0.59	0.57
Three-way Interaction	12	70	1.43	0.83	0.73	0.90	0.36	0.59	**1.94**

Table 4 Final model from ANCOVA on percent predation using Principal Components 1 through 7 as covariates and sampling dates, genetic lines, trichome phenotype and their interactions as "fixed" effects. For each effect, the numerator and denominator degrees of freedom, *F*-statistic, and probability are shown. The slopes also are shown for the seven covariates. Statistically significant effects are shown in bold; non-significant interactions were removed from the final model

Effect	Numerator df	Denominator df	*F*-statistic	*P*	Slope
PC 1	1	173	4.70	**0.031**	4.41
PC 2	1	173	0.17	0.684	1.20
PC 3	1	173	3.39	0.067	−5.50
PC 4	1	173	0.75	0.387	−2.57
PC 5	1	173	3.55	0.061	−6.67
PC 6	1	173	2.01	0.157	−6.11
PC 7	1	173	0.18	0.667	−1.97
Sampling Date	4	173	21.49	**<0.001**	
Genetic Line	3	173	0.80	0.498	
Trichome Phenotype	1	173	0.17	0.685	
Date by Line	12	173	2.04	**0.023**	
Date by Trichome Phenotype	4	173	3.16	**0.016**	
Line by Trichome Phenotype	3	173	3.83	**0.011**	

Although we utilized genetic lines of plants that differed in inducible VOC production in the greenhouse, there were no season-long differences in predation among genetic lines. Predation, PC 1 (general production) and PC 3 (largely contrasting (*E*)-β-caryophyllene with (*E*)-β-ocimene), however, all varied due to the date by genetic line interaction, as might be expected due to the seasonal variation in VOC production overall. Only a few studies to date compare either the ability of plant genotypes to attract natural enemies or VOC production in both the laboratory and field. Results from laboratory and field studies concur in some cases but not in others. Preference studies on *Cotesia* sp. attracted to volatiles produced by different cultivars of *Brassica oleraceae* in the laboratory reliably predicted differences in parasitization of herbivores on those cultivars in the field (Poelman et al., 2009). In *Trifolium repens,* however, fewer individual components were detected in field-grown than in laboratory-grown plants, and the emission of some compounds declined in response to increasing herbivory in the field but increased in response to herbivory in the laboratory (Kigathi et al., 2009). These authors argued that the release of volatiles from plants in the field was influenced by additional unmeasured biotic and abiotic factors that were absent in the laboratory and recommended caution in extrapolating profiles from VOC production to plants growing in the field. The results with *D. wrightii* additionally suggest caution in extrapolating results from plant genotypes assayed at a single developmental stage to potential differences over their full growing season.

The greenhouse experiments showed a greater effect of glandular trichomes on predation by *G. pallens* than did the field experiments. The major products of the glandular trichomes are esters of glucose and straight-chain organic acids (van Dam and Hare, 1998a). In *D. wrightii,* these acylsugars largely act as deterrents against the herbivores of *D. wrightii* as well as the natural enemies of those herbivores, but not as toxins to either group (Gassmann and Hare, 2005; Hare, 2005). There can be substantial environmental variation in acylsugar production under field conditions, however (Forkner and Hare, 2000; Hare, 2005). Although no direct comparisons of acylsugar production were made in this study, previous research has shown that acylsugar production has been higher in the greenhouse (ca. 0.8 μmol·cm^{-2}, Hare and Walling, 2006) than in the field (ca. 0.1–0.5 μmol·cm^{-2}, Hare, 2005). If generally true, then it is possible that the use of greenhouse-grown plants may overestimate the impact of glandular trichomes on predation by *G. pallens* because of the greater acylsugar production in the greenhouse environment.

One area of controversy in the study of HIPVs concerns the specificity and complexity of HIPV blends. The cases in which successful biological control has been achieved by the importation and release of specialist natural enemies against introduced crop pests in conventional agroecosystems argues in favor of herbivore suppression by specialized natural enemies, and, by inference, the emission of specific HIPV blends by plants to attract those specialists. Such systems, however, may be inappropriate models in which to understand how herbivore populations are regulated in natural systems (Hawkins, 1992; Hunter and Price, 1992). Instead, for native herbivore populations in their native environments, generalist predators more frequently impose top-down suppression of herbivores than do specialist parasitoids (Hawkins et al., 1999). As linear systems of crops, a single introduced herbivore species, and a single species of specialized natural enemy may be inappropriate models to understand how herbivore populations are regulated in natural systems, then such linear systems also may be inappropriate models to infer how HIPV-mediated indirect defenses of plants may have evolved (Hare, 2011). For generalist predators like *G.*

pallens, which preys on several species within *D. wrightii*'s herbivore community (Gassmann and Hare, 2005), it may not be necessary for the predator to identify the species of herbivore, and complex and herbivore-specific VOC blends may not be necessary to attract *G. pallens* to plants damaged by any suitable prey species. Similarly, the fact that *G. pallens* is known to respond to individual compounds when presented in isolation (Kessler and Baldwin, 2001; James, 2003, 2005; Halitschke et al., 2008) may constrain or eliminate the possibility of *G. pallens* being differentially attracted to herbivore-damaged plant genotypes that produce different HIPV blends and, ultimately, imposing natural selection on plant genotypes for the production of any particular blend.

In view of the rapid colonization of plants by both herbivores and natural enemies (Elle and Hare, 2000; Hare and Elle, 2002), it may not be necessary for *D. wrightii* to produce any strong "long-range" cues except perhaps in the early spring, when some plants are colonized by herbivores but most are not. It would be tempting, therefore, to conclude that the seasonal pattern of VOC production by *D. wrightii* has been optimized by natural selection so that plants produce these cues in large amounts only when the cues greatly increase predation. Such a conclusion depends upon a number of untested assumptions, however. The first is that high levels of VOC production in response to herbivory through the remainder of the season are detrimental and selected against. One possibility is that these compounds have such high allocation costs that reproduction would be reduced due to the allocation of photosynthate away from reproduction to HIPV production when HIPVs were no longer needed. Unfortunately, we know of no study showing that HIPV production imposes a fitness costs on plants (see also Kessler and Heil, 2011).

Another possibility is that there may be ecological costs later in the season associated with high levels of HIPV production that counterbalance their potential benefits. Possibly HIPVs induced in *D. wrightii* may attract other herbivorous insects, as is known to be the case for HIPVs of the host plants of the Colorado potato beetle (Landolt et al., 1999), Japanese beetles (Loughrin et al., 1995), and flea beetles (Halitschke et al., 2008). Although no similar studies have been carried out to determine if additional herbivores also are attracted to *D. wrightii* damaged by *L. daturaphila*, aspects of the life history of both *D. wrightii* and *L. daturaphila* may call into question whether continued high levels of HIPV production by *D. wrightii* throughout the season imposes a significant ecological cost mediated by attraction of additional individuals of *L. daturaphila* or other herbivore species to insect-damaged plants. Individual *D. wrightii* plants within patches quickly become colonized by overwintering herbivores in the early spring, then suffer chronic herbivory throughout the growing season (Elle and Hare, 2000; Hare, 2010). Because of the rapid colonization of plants and chronic, season-long herbivory, the ecological significance of additional herbivores being preferentially attracted to damaged plants would seem to be short-lived.

Another type of ecological cost that may account for the seasonal decline in HIPV production is based upon the observation that volatiles induced by herbivores repel potential pollinators. In the allogamous species, *Solanum peruvianum*, pollinators visited fewer flowers and spent less time at flowers of herbivore-damaged plants than undamaged plants (Kessler and Halitschke, 2009). If this is a general phenomenon, then it is at least plausible that there could be evolutionary or physiological conflicts between the competing processes of attracting the natural enemies of herbivores vs. attracting pollinators. The magnitude of such conflicts may vary among plant species with different mating and pollination systems. Such conflicts may be less important for autogamous species like *D. wrightii*, in which ca. half of the seeds produced by flowers may be due to self-pollination in absence of pollinators (Elle and Hare, 2002; Bronstein et al., 2009) than for species that absolutely require insect pollinators for reproduction.

Alternative explanations exist for the production of inducible VOCs by plants. One such explanation is that the production of such compounds alleviates certain abiotic stresses. High temperatures generally increase VOC production within broad limits (Holopainen and Gershenzon, 2010), and limited research shows that the emission of HIPVs also are temperature-dependent. In maize, total HIPVs increased with temperature from 17–27°C but were reduced at 37°C; the quantities of emissions at 37°C were not significantly different from those at 17°C or 22°C (Gouinguene and Turlings, 2002). At still higher temperatures (ca. 40–45°C) then, terpene emissions may be inhibited in other plant species (Loreto and Schnitzler, 2010), but the ambient temperatures during the volatiles collection period in this study were never that high (Table 1). A hypothesis of temperature inhibition certainly could not account for the low levels of VOC production in August, when temperatures during the collection period were relatively cool. Previous research also has shown that local summer temperatures are no barrier to VOC production for plants that are sufficiently young (Hare, 2010).

Finally, such seasonal patterns may reflect general processes of plant development and internal source-sink relationships as the plants transition from vegetative growth to reproduction (Herms and Mattson, 1992) independently of seasonal variation in herbivory and herbivore-natural-enemy interactions. Such patterns also may reflect general patterns of plant development and not patterns specific to *D. wrightii*. Given the high similarity of the induced defenses of different solanaceous species, it is likely that those defenses are homologous traits inherited from a

common ancestor and not novel, species-specific adaptations. These homologous traits no doubt have gone through ecological and evolutionary diversification among extant sister clades even as the life histories of species within those clades also have diverged. Thus, the role of phylogenetic legacy in governing for the seasonal patterns of VOC production in *D. wrightii* and potentially other species also must be considered along with the adaptive explanations above.

In summary, the greenhouse experiments showed that predation of *L. daturaphila* on young *D. wrightii* plants by *G. pallens* depended upon the interaction between trichome phenotype and induction status in a predictable way: in the greenhouse, induction or damage increased predation of larvae on velvety plants more than on sticky plants. The field experiments, however, revealed a more dynamic series of results that were strongly influenced by season and ecological context. High levels of VOCs in response to plant damage facilitated predation especially in the spring, but high levels of predation also occurred on plants that emitted lower levels of VOCs later in the summer. VOC production may facilitate the colonization of herbivore-infested plants and the discovery of prey by predators in the spring, but western big-eyed bugs apparently can discover and attack prey using other senses such as vision (Eubanks and Denno, 2000) when VOC production declines. It is unclear why or how VOC production in *D. wrightii* declines with plant development, but, because of the nonspecific association between *G. pallens* and either *D. wrightii* or *L. daturaphila*, the seasonal decline in VOC production more likely is incidental to the interaction between *L. daturaphila* and *G. pallens* rather than because of that interaction. Future research to better understand the ecological significance of inducible VOC production and other inducible defenses in the Solanaceae may benefit through comparative studies of several different species that exhibit a range of life history traits in their natural environments.

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Differential Activity of Multiple Saponins Against Omnivorous Insects with Varying Feeding Preferences

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Abstract A variety of glycosylated and unglycosylated saponins from seven different plant families (Aquifoliaceae, Asparagaceae, Caryophyllaceae, Dioscoreaceae, Leguminosae, Rosaceae, Sapindaceae) were tested against the corn earworm, *Helicoverpa zea*, and the fall armyworm, *Spodoptera frugiperda*. The corn earworm feeds readily on both monocots and dicots, while the fall armyworm is primarily a grass feeder. Most of the saponins were similarly effective or ineffective against both insect species, with the glycosides being the primary active form (compared to aglycones). However, one aglycone possessed antifeedant properties toward the fall armyworm. Thus, in contrast to many plant secondary metabolites effective against either of these two species where the aglycone is more effective, in the case of the saponins the opposite is generally true. This appears to be a contradictory strategy of plant defenses that requires further consideration. The activity of protodioscin against insects is reported for the first time and may be important in insect defense by the bioenergy crop switchgrass.

Key Words *Helicoverpa* · *Spodoptera* · Soyasaponin · Quillaja · Mate · Plant defense

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Introduction

A variety of plant secondary chemicals can act as defenses against insects, including alkaloids, flavonoids, glucosinolates, and phenolic acids (Rosenthal and Janzen 1979). An additional group of compounds active against insects are the saponins, which have diverse chemical structures typically consisting of a triterpenoid or steroidal nucleus with a variety of glycosylation patterns (Hostettmann and Marston, 1995). Saponins are found in over 100 different plant families, although they often are more common in species from specific families or genera, such as Leguminosae and Liliaceae (Harborne and Baxter, 1993). As such, a wide variety of specialist and generalist insects would be expected to encounter them.

Initial information on activity of saponins against insects was limited to reports of leguminous sources and extracts (Applebaum and Birk, 1979). Later work indicated several high saponin plant parts from diverse families, including Aquifoliaceae and Theaceae in addition to Leguminosae, are resistant to insects (Hostettmann and Marston 1995). Since then, the number of studies showing activity of concentrated saponin fractions or pure saponins against insects has greatly expanded, and affected insects include aphids, beetles, caterpillars, and flies (De Geyter et al., 2007). However, comparative studies of the effects of multiple saponins of diverse sources against insects of different feeding specialization is still limited.

The corn earworm, *Helicoverpa zea*, feeds on plants in a variety of families, including Gramineae, Leguminosae, Malvaceae, and Solanaceae (Metcalf and Metcalf, 1995). The fall armyworm, *Spodoptera frugiperda*, also is an omnivorous caterpillar, but specializes more on Gramineae (Metcalf and Metcalf, 1995). The activity of saponins from a variety of host and nonhost plants against these two insect

species were evaluated against first instar caterpillars, and differential effects were noted, as reported herein.

Methods and Materials

Insects Corn earworms and fall armyworms were reared at 27±1°C., 50±10% relative humidity and a 14:10 h L:D photoperiod on pinto bean diet as described previously (Dowd, 1988). First instars were used in bioassays.

Chemicals (Structures Fig. 1, plant host sources Table 1). *Gypsophila* saponins (S1252, lot 6F0539, original source *Gypsophila*)(Dowd, 1989), quillaja bark saponin (S4521, lot 57H9524), and oleanic acid, 97% (original source *Saponaria officianalis*) were obtained from Sigma Chemical Company. Due to their complexity (Guo et al., 2000) the quillaja bark saponins were not purified further, but characterization using HPLC and MS determinations described below indicated they consisted of several dozen saponins, mainly consisting of quillaic acid aglycone (Fig. 1) with different combinations of galactose, glucuronic acid, and xylose at position 3 and apiose, rabinose, fucose, rhamnose, and xylose at position 28, with approximately 25% sapogenin aglycones (total of approxmately 80% saponins); a composition consistent with prior reports of this material (Chapagain and Wiesman, 2006). Using the same methodology to characterize the gypsoside preparations, total saponin content was greater than 80% with 4 main glycosides, including at least one gypsogenin derivative, (probably gypsoside A), saponeside D, and quilliac acid desmosides, based on prior reports of composition of a similar material (Higuchi et al., 1985; Frechet et al., 1991) and our available HPLC and MS analysis. Caffeine, diosgenin, glycyrrhizic acid, and protodioscin were obtained from ChromaDex (Irvine, CA, USA) and were 100, 96.0, 95.6, and 95% pure, respectively. Sapogenol B and B group soyasaponins were obtained from soybean seeds (Berhow et al., 2006), and final purity was over 95% for the aglycones (consisting of 90% of sapogenol B and minor amounts of sapogenol A and E) and glycoside mix (see below and Fig. 1). Mate (yerba mate, *Ilex paraguariensis*) leaves were obtained from a local distributer, and ursolic saponins were purified to greater than 95%

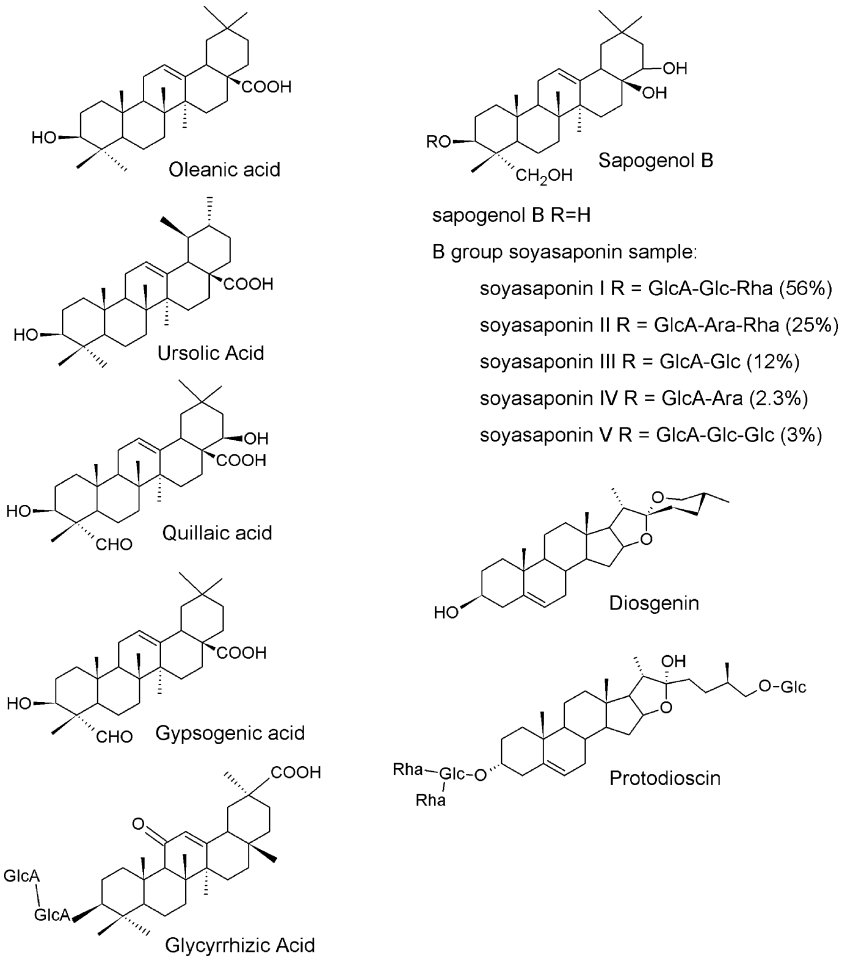


Fig. 1 Sapogenins and saponins tested against corn earworms (*Helicoverpa zea*) and fall armyworms (*Spodoptera frugiperda*). See Methods and Materials for further descriptions

Table 1 Saponins tested and plant sources

Saponin	Major source	Family	Tissue
diosgenin	Mexican wild yam	Dioscoreaceae	root
glycyrrhizic acid	licorice	Leguminosae	root
Gypsophila saponins	baby's breath	Caryophyllaceae	root
oleanic acid	soapberry	Sapindaceae	fruit
protodioscin	aspargus	Asparagaceae	shoot
quillaja acid saponins	quillaja	Rosaceae	bark
B group soyasaponins	soybean	Leguminosae	leaves/seed
sapogenol B	soybean	Leguminosae	leaves/seed
ursolic acid saponins	yerba mate	Aquifoliaceae	leaves

Compiled from Hostettmann and Marston, 1995; Lacaille-Dubois, 2000; Ganzera et al., 2001; Wang et al., 2003.

glycosides by methods similar to those used for the soyasaponins, and structures were identified by LC-MS (below). Purified saponins consisted of a mixture of 7 glycosides, and included mate saponin 1 and 2, plus 5 additional uncharacterized saponins, but no mate saponin 3, 4, or 5.

Chemical Separation and Purification of Saponins from Extracts

A Buchi (Newcastle, DE, USA) Sepacore flash chromatography system with dual C-605 pump modules, C-615 pump manager, C-660 fraction collector, C-635 UV photometer, with SepacoreRecord chromatography software was used. A Buchi C-670 Cartridger system was used to load 40× 150 mm flash columns with approx. 90 g of preparative C-18 reverse phase bulk packing material (125 A, 55–105 μm, Waters Corp., Milford, MA, USA). Columns were installed in the flash chromatography system and equilibrated with 30% acetonitrile and 70%, 0.5% acetic acid (in water) for 5 min at a flow rate of 30 ml per min. After samples (10–15 ml) were injected, the column was developed to 100% acetonitrile over 20 min, and the effluent was monitored at 210 nm. Fractions based on absorbance were collected in the fraction collector controlled by the software program. Fractions containing saponins were determined by analytical chromatography and LC-MS. After evaporation to remove the organic solvent, saponins were recovered as white solids by precipitation/crystallization or freeze-drying.

Further purification was achieved by reverse phase preparative liquid chromatography. A Shimadzu (Columbia, MD, USA) preparative HPLC system was used with dual 8A pumps, SIL 10 vp autoinjector, SPD M10Avp photodiode array detector, SCl 10Avp system controller all operating under the Shimadzu Class VP operating system. Ten ml of sample aliquots in methanol were injected on a Phenominex (Torrence, CA, USA) Luna C18(2) semi-preparative reverse phase column (10 μ, 100A, 250×50 cm). The column was pre-equilibrated with 1% acetic acid, 30% acetonitrile and 69% water at a flow rate of 50 ml per min, and the effluent was monitored at 210 nm. The column was developed to 70% acetonitrile over 45 min. UV absorbing peaks were collected by hand. The procedure was repeated to obtain sufficient purified material. Pooled material was allowed to evaporate, and the resulting fractions were evaluated by LC-MS for saponin content.

HPLC analysis was conducted on a Shimadzu LC-20 HPLC system (LC-20AT quaternary pump, DGU-20A5 degasser, SIL-20A HT autosampler, and a SPD M20A photodiode array detector, running under Shimadzu LC solutions version 1.22 chromatography software, Columbia, MD, USA). The column used was an Inertsil ODS-3 reverse phase C-18 column (5 μM, 20×4.6 mm, with a Metaguard column, from Varian). For saponin analysis, the initial conditions were 30% acetonitrile and 0.025% trifluroacetic acid (TFA) in water, and a flow rate of 1 ml per min. The effluent was monitored at 210 nm with the detector. After injection (typically 20 μl), the column was developed to 50% acetonitrile and 0.025% TFA in a linear gradient over 45 min. Standard curves based on nanomoles injected were prepared from a characterized mix of B group soyasaponins previously prepared (Berhow et al., 2006). The molar extinction coefficient for soyasaponin I was used to quantitate the mate saponins. The identities of the mate saponins were confirmed by purification and LC-MS.

For LC-MS analysis, samples were run on an Applied Biosystems/MDS Sciex Qstar Elite Q-TOF mass spectrometer with a Turboionspray electrospray source, and an Agilent 1100 series HPLC system (G1379 degasser, G1357A binary capillary pump, G1389A autosampler, G1315B photodiode array detector, and a G1316A column oven) all running under Applied Biosystems Analyst 2.0 (build 1446) LC-MS software. The MS was calibrated at least daily with a standard calibration mixture recommended by Applied Biosystems, and the signal detection was optimized as needed. Data were acquired with the MOF MS mode, negative. The MS parameters were as follows:

accumulation time—1 sec, mass range—2,100–1,000 daltons, source gas—1–50 units, source gas 2–35 units, curtain gas—25 units, ion spray voltage—4,500, source heater—400°C, declustering potential—80, focusing potential—265, declustering potential 2–15, ion release delay—6, ion release width—5. The column was a 3×150 mm Inertsil reverse phase C-18, ODS 3, 3 μm column (Metachem, Torrence, CA, USA) with a Metaguard guard column. Solvent systems were A: water with 0.25% formic acid, and B: acetonitrile. The column was equilibrated with 30% B at a flow rate of 0.25 ml per min. After injection (15 μl), the column was developed with a linear gradient to 80% B over 60 min. Column effluent was monitored at 210 nm in the PDA detector. The software package was set to collect mass data between 150–1,000 AMUs. Generally, the most significant sample ions generated under these conditions were [M-1]- and [M + HCOO]-.

Bioassays: Various Saponins Initially, chemicals were absorbed into freeze-dried diet disks in either ether, acetone, or water solutions at a rate of 25 μl per 15 mg disk to yield a final concentration of 1% when the disk was hydrated, using assay setups as described previously (Dowd et al., 2007). When acetone or ether was used as a solvent, the disks were placed in a fume hood for 10 min to evaporate solvent. Water was added at a rate of 25 μl per disk. For water solubilized preparations of saponins, liquid was added directly to the disk. Disks were allowed to equilibrate for 2 h prior to bioassay setup. At least 10 individual caterpillars were set up in each saponin or solvent treatment. Assays ran for 2–3 d, depending on whether cannibalism was noted (which is more of a problem with corn earworms). At the end of the assay period, caterpillars were frozen, and later weighed using a Mettler AE163 analytical balance, which has a precision of 0.01 mg.

Larger volume heated diet incorporation also was used to further examine activity of several of those compounds with significant activity at 1% (*Gypsophila*, mate, and quillija saponins, sapogenol B and B group soyasaponins). Assays were run with a lower concentration of each saponin (1,000 ppm) for 7 d. Materials were dissolved in 125 μl of water or acetone, and added to warm, still liquid agar as described previously (Dowd, 1988). After solidifying, diets with acetone were placed in a fume hood for 1/2 h in order for the solvent to dissipate. Corn earworms and fall armyworms were caged separately in this case using 24 well plates (Dowd, 1988). For 10 d-assays with quillija saponins at 1,000 ppm, diet prepared as described above was dispensed into 35 ml plastic cups, and larvae again were individually added to the cups. At the end of the assay period, caterpillars were frozen and weighed as described above.

Significant differences between survivor weights of caterpillars feeding on saponin containing diets and corresponding solvent control diets were determined by analysis of variance using SAS Proc GLM statistical analysis software Version 8.0 (SAS Institute, Cary, NC, USA).

Bioassays: Sapogenol B Due to the activity noted for the acetone soluble sapogenol B against fall armyworms (Results), it was possible to set up choice assays. Choice assays were performed using maize (*Zea mays*) leaves (which do not contain saponins—see Discussion) from the 10th position of a 12 leaf plant from four inbred B73 plants grown as described previously (Dowd et al., 2007). Three paired sections of each leaf were used, and each assay pair consisted of 4 cm^2 sections from opposite sides of the midvein. Sapogenol B was dissolved in acetone at a concentration that would yield a 1,000 ppm concentration once it was added to both sides of the leaf. For each side of a leaf piece, 100 μl of acetone or acetone solution of soyasapogenol B were added to a side, and the acetone was allowed to evaporate between applications. The paired leaf sections were placed in a 5 cm Petri dish as described above, along with a 4.25 mm piece of Whatman #1 filter paper (Whatman International, Maidstone, England) to which 120 μl of sterile distilled water were added. Twenty first instars were added to each dish, and feeding on each leaf section was rated by counting 0.25 mm^2 hole (approx. head capsule size) equivalents for 2 d, as described previously (Dowd et al., 2007).

Data were analyzed for significant antifeedant effects using a paired *t*-test (within SAS Proc Means).

Results

In only one case was mortality noted (11.1% for corn earworms fed mate in diet for 2 d), so reported results are limited to weights of larvae fed respective diets. Although none of the saponins was as active as caffeine, some showed significant activity against corn earworm and/or fall armyworm larvae after 2–3 d at 1% in diet disks (Table 2). The *Gypsophila* saponins, protodioscin, and the quillija saponins were similar in activity towards both insect species, and inhibited growth by approximately 20–50%. The quillija saponins appeared somewhat more active than *Gypsophila* saponins or protodioscin towards both species based on the degree of reduction in growth rates they caused. The mate saponins were more active towards the corn earworm (50.6% control weight) than the fall armyworm (75.4% control weight), while diosgenin and glycyrrhizic acid had no significant activity towards either species tested compared to solvent control diet. Interestingly,

Table 2 Saponin (1%) activity against first instar larvae of *Helicoverpa zea* (corn earworm) and *Spodoptera frugiperda* (fall armyworm)

Compound	Percent of control weight			Assay days
	Fall armyworm	Assay days	Corn earworm	
water	100.0	3	100.0	2
caffeine	26.9*	3	14.7*	2
Gypsophila saponins	76.6*	3	80.4	2
mate saponins	75.4	2	50.6*	2
protodioscin	70.6*	3	71.2*	2
quillaja saponins	60.9*	3	56.9*	2
B group soyasaponins	108.6	3	74.5*	2
acetone	100.0	3	100.0	2
diosgenin	96.2	3	93.2	2
glycyrrhizic acid	90.2	3	90.9	2
sapogenol B	66.4*	3	118.4	2
ether	100.0	3	100.0	2
oleanic acid	92.2	3	90.9	2

Values followed by an "*" were significantly different from corresponding solvent control values at $P<0.05$ by analysis of variance.

B group soyasaponins were significantly active against corn earworms (74.5% control weight) while the aglycone (sapogenol B) was not; the opposite was true for fall armyworms (larvae fed diet containing sapogenol B weighed 66.4% those fed control diet).

For the compounds run at the lower dose rate (1,000 ppm) over a 7 d period of time, only quillija saponins showed a statistically significant effect, and only on fall armyworms (74.3% control weight, $F=9.45$, $P=0.004$). There also was a trend for fall armyworms to be smaller when fed diet containing *Gypsophila* saponins at 1,000 ppm (80.9% control weight) similar to that noted for the 1% dose range of *Gypsophila* saponins. In the 10 d assay run with the quillaja saponins, most larvae that fed on the control diet reached the fifth (last) instar, but most larvae fed on the treated diet only reached the fourth instar. Corn earworms fed the quillaja saponin containing diet were 72.7% the weight of those fed control diet ($F=17.39$, $P<0.001$). Results with fall armyworms were similar to those noted with the corn earworms, as those larvae fed diet with 1,000 ppm of quillija saponins weighed 51.7% of those fed solvent control diet ($F=20.96$, $P<0.001$).

Sapogenol B appeared to have antifeedant properties toward fall armyworm larvae when applied to maize leaves. On day 1 of the assay, the mean difference in feeding on sapgenol-treated leaves and controls was $14.3\pm4.2\text{mm}^2$ ($t=3.38$, $P=0.006$) and on day 2 the mean difference was $49.2\pm9.2\ \text{mm}^2$ ($t=5.32$, $P<0.001$).

Discussion

Various plant saponins have insecticidal activity against flies, beetles, caterpillars, and aphids (De Geyter et al., 2007). We found several saponins with insecticidal activity against the caterpillar pests *H. zea* and *S. frugiperda*. Some we tested also are active against other species of insects. Levels of a gypsoside glycoside have been correlated with resistance of yellow rocket *Barbarea vulgaris* to the flea beetle *Phyllotreta nemorum* (Nielsen et al., 2010). Quillija saponin extracts are active against mosquito larvae (Pelah et al., 2002; Wiesman and Chapagain, 2003) aphids (*Acyrthrosiphum pisum*) and caterpillars (*Spodoptera littoralis*) (De Geyter et al., 2007). We also noted activity of sapogenol B against fall armyworms, a compound that is inactive compared to some other alfalfa saponins to *S. littoralis* (Adel et al., 2000). Green holly (*Ilex opaca*) fruits, which have high levels of saponins compared to ripe fruits, were more toxic to larvae of *S. frugiperda* than ripe fruits (Kreuger and Potter, 1994). To our knowledge, the activity of protodioscin and mate saponins against insects is reported here for the first time.

The saponins we tested can occur at ranges of up to 22%, but are typically 0.1–1% dry weight (Shi et al., 2004). While doses of saponins we used initially were somewhat higher than those naturally occurring in leaves (e.g., B group soyasaponins, protodioscin), results based on bio-assays with artificial diet and saponins may lead to an underestimaion of the bioactivity of saponins, since larvae do not need to cope with the stress factors typically encountered additionally in natural plant sources (Smith et al., 1994). Longer term studies also have indicated significant effects on growth and developmental rates at lower concentrations (e.g., Adel et al., 2000). We also noted more significant effects when exposing larvae to the compounds (such as quillija saponins) over a longer period of time. The antifeedant effects we noted with fall armyworms and sapogenol B may be due to an interference in

the ability to perceive sweet compounds, as has been reported for the gymnemic acid saponins (Eisner and Halpern, 1971).

Typically, plant secondary metabolites are more active toward insects as the aglycones than they are as the glycosides, including cardiac glycosides, cyanogenic glycosides, flavonoids, glucosinolates, iridoid glycosides, phenolics, sesquiterpene lactones, and triterpenoid glycosides (e.g., Dowd et al., 1983; Spencer, 1987). For saponins, the type of sugar attached to the saponin determined the effectiveness against *S. littoralis* (Adel et al., 2000). Another study indicated saponins need both a hydrophilic and a lipophilic portion for maximum activity against *S. littoralis* (Saha et al., 2010). These prior findings may explain some of the differences in activity of the saponins tested in the present study. However, the requirements for saponin toxicity to the two insect species examined in this study appear to be more complicated, as some of the saponins were similar in activity against *H. zea* and *S. frugiperda*, while others were quite different. Notably, the sapogenol B aglycone was more active against *S. frugiperda* than against *H. zea*, while the opposite was true for the glycosylated form.

Despite the different host ranges of the two moth species examined, responses were similar except for the B group soyasaponins vs. sapogenol B, and to some degree, for mate saponins. Based on prior reports of plant host ranges for the corn earworm and fall armyworm (Metcalf and Metcalf, 1995) and the plant tissues where the saponins we analyzed are located (see Table 1), we suspect the insect species we tested are not exposed to these compounds in nature. The exceptions are the saponins from soybean, baby's breath (*Gypsophila*), and asparagus. However, these saponins had activity against the corn earworms and fall armyworms that was comparable to the activity of the saponins from quillaja and yerba mate, which are not host plants. Future studies need to investigate whether insects are less able to develop resistance to saponins compared to other classes of plant defensive compounds.

The presence of saponins in plants has implications beyond conferring insect resistance. Practically, saponins from *Quillaja saponaria* (USEPA PC code 097095) and *Chenopodium quinoa* (USEPA PC code 097094) have been registered as biopesticides for fungal control (USEPA 2002, 2007). Saponins have been implicated as important resistance molecules targeted at plant pathogens, but typically are absent in modern lines of grain crops (Papadopoulou et al., 1999). Our results suggest they may also be important in insect resistance in different plants for two major insect pests which feed on grain crops such as maize, as several different forms reduced growth rates in both of the lepidopteran species tested. Hence, enhancement of saponins may provide multiple resistance benefits against both insects and plant pathogens. However, the plants that may be bred or engineered for enhanced insect resistance through presence/increases in appropriate saponin levels need to be considered for end uses, as some saponins can adversely affect ruminants (Flaoyen, 2000, Francis et al., 2002), while others can even improve animal health (Francis et al., 2002). A number of reports also have indicated human health benefits from a variety of saponins (Rao and Gurfinkel, 2000; Shi et al., 2004, Güçlü-Üstünda and Mazza, 2007), suggesting that pest resistance and human health benefits can be promoted by use of these molecules. Selective increases in saponin content of soybean leaves suggest that increased resistance to the soybean pest *H. zea* could occur. Presence of saponins in switchgrass (Lee et al., 2009) suggests not only implications in insect resistance, but also for bioenergy, as fermentation byproducts may contain useable saponins for steroid precursors (Harborne and Baxter, 1993) or insecticidal/fungicidal extracts.

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Distinguishing Defensive Characteristics in the Phloem of Ash Species Resistant and Susceptible to Emerald Ash Borer

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Abstract We examined the extent to which three *Fraxinus* cultivars and a wild population that vary in their resistance to Emerald Ash Borer (EAB) could be differentiated on the basis of a suite of constitutive chemical defense traits in phloem extracts. The EAB-resistant Manchurian ash (*F. mandshurica*, cv. Mancana) was characterized by having a rapid rate of wound browning, a high soluble protein concentration, low trypsin inhibitor activities, and intermediate levels of peroxidase activity and total soluble phenolic concentration. The EAB-susceptible white ash (*F. americana*, cv. Autumn Purple) was characterized by a slow wound browning rate and low levels of peroxidase activity and total soluble phenolic concentrations. An EAB-susceptible green ash cultivar (*F. pennsylvanica*, cv. Patmore) and a wild accession were similar to each other on the basis of several chemical defense traits, and were characterized by high activities of peroxidase and trypsin inhibitor, a high total soluble phenolic concentration, and an intermediate rate of wound browning. Lignin concentration and polyphenol oxidase activities did not differentiate resistant and susceptible species. Of 33 phenolic compounds separated by HPLC and meeting a minimum criterion for analysis, nine were unique to Manchurian ash, five were shared among all species, and four were found in North American ashes and not in the Manchurian ash. Principal components analysis revealed clear separations between Manchurian, white, and green ashes on the basis of all phenolics, as well as clear separations on the basis of quantities of phenolics that all species shared. Variation in some of these constitutive chemical defense traits may contribute to variation in resistance to EAB in these species.

Key Words *Agrilus* · Ash · Defense proteins · Emerald Ash Borer · *Fraxinus* · Phenolics · Constitutive resistance

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Introduction

Qualitative and quantitative variation in chemical defenses has long been associated with variation in the resistance of plants to particular attackers (Levin, 1976). While relationships between host-plant defenses and herbivore attack rates have been relatively well studied in the foliage of herbaceous and woody plants, and for conifer/bark beetle interactions, less is known about defenses of angiosperm trees to wood-boring herbivores (e.g., Dunn et al., 1990). Our lack of understanding of such relationships is of particular concern in the face of the increased number of invasive wood-boring insects that have emerged in novel habitats in recent years.

Larvae of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae; EAB), feed on phloem tissue of ash trees (*Fraxinus* spp.). In its home range in Eastern Asia, EAB utilizes several ash species, including Manchurian ash (*F. mandshurica*), but it typically attacks

(and sometimes kills) only stressed ash trees (Gould et al., 2005; Liu et al., 2007). In its introduced range in North America, EAB also utilizes several species of ash, including white (*F. americana*) and green (*F. pennsylvanica*) ash, but even healthy trees are susceptible to attack (Poland and McCullough, 2006). In a common garden study in Michigan near the core infestation zone of EAB, white and green ash cultivars were attacked more heavily by EAB and were more likely to die from attack than a Manchurian ash cultivar (Rebek et al., 2008). Some of this variation may be attributable to as yet undetermined adult oviposition preferences (Pureswaran and Poland, 2009), but these findings suggest that physical and biochemical defense mechanisms of the phloem may limit the feeding, growth, and survival of EAB larvae in Manchurian ash. Mechanisms of resistance to wood-boring insects of angiosperm trees have not been well studied, but they likely involve antibiotic secondary metabolites common in woody tissues (e.g., phenolics), defensive proteins in the phloem, and physical defenses such as sap flow to wounded areas and the capacity to rapidly heal wounds (e.g., Dunn et al., 1990; Wainhouse et al., 1990, 1998; Kehr, 2006).

We hypothesized that the susceptibility of North American ashes may stem from a lack of an evolutionary history with EAB, and an inability to defend against larval feeding with appropriate chemical defenses (Rebek et al., 2008). Thus, we predicted that Manchurian ash would constitutively express either higher levels of chemical defenses or a unique profile of defenses in the phloem compared to North American ashes. In a companion paper, we showed that the profile of constitutive phenolic secondary metabolites in phloem from bare-root dormant stems of a cultivar of Manchurian ash was distinguishable from white and green ash cultivars by its unique content of hydroxycoumarins (particularly fraxin, mandshurin, and fraxidin hexoside), calceolariosides A and B, and significantly higher concentrations of pinoresinol glucoside and pinoresinol, along with some other minor differences (Eyles et al., 2007). While the factors specifically conferring resistance to EAB in Manchurian ash remain elusive, these sorts of compounds are potentially involved.

Here, we compared the constitutive activities of three defense proteins, lignin and total soluble protein content, and phenolic profiles in the phloem of actively growing Manchurian, white, and green ash cultivars, and in green ash originating from a wild population. Use of a wild population of green ash allowed comparisons of its chemistry to that of a cultivated variety. Although little studied in relation to wood borer feeding, defensive proteins in the phloem are recognized as potentially important resistance factors to phloem-feeding insects (Kehr, 2006). Polyphenoloxidase and peroxidase activity are associated with resistance of plants to both insects and pathogens (Felton et al., 1989; Dowd and Norton, 1995; Cipollini and Redman, 1999; Cipollini et al., 2004), presumably by increasing the toxicity of phenolics or by increasing cell wall toughness. Trypsin inhibitor activity may slow herbivore growth and feeding by decreasing protein assimilation by inhibiting the action of serine proteases, and thus slow larval growth rate (e.g., Cipollini et al., 2004). Recently, transcripts of serine proteases have been found in the midgut of EAB larvae (Mittapalli et al., 2010). We also examined the rate at which phloem extracts browned due to the action of constitutive phenoloxidases on endogenous soluble phenolics present in the extract, which represents the generation of toxic quinones and other polyphenolics that can be associated with resistance and wound healing (Cheng and Crisosto, 1995). Finally, we examined constitutive levels of total phenolics and individual phenolic metabolites by HPLC, as in Eyles et al. (2007), but in this work we examined both qualitative and quantitative variation in expression of phenolic metabolites in actively growing stems sampled during a time when EAB larvae would be active, as opposed to sampling dormant stems.

Methods and Materials

Plant Material and Growth Four-year-old saplings of Manchurian, white, and green ash were planted in a common garden on the campus of Wright State University in May 2007. We grew 8 individuals of Manchurian ash (cv. Mancana), 11 individuals of white ash (cv. Autumn Purple), 11 of green ash (cv. Patmore), and 11 of green ash grown from seed from a wild population. The ash cultivars were obtained from Bailey Nurseries (St. Paul, MN, USA), and the wild green ash was supplied by the USDA Forest Service Northern Research Station (Delaware, OH, USA). The cultivars used were the same as those used in studies of phenolic chemistry by Eyles et al. (2007) and of field resistance to EAB by Rebek et al. (2008), but neither of those studies included plants from any wild populations. Trees were planted in a grid spaced approximately 3 m from each other, with mowed grass in between. Tree trunks were protected from deer and other mammal damage with 1 m tall plastic tree guards. All trees were watered three times throughout the first growing season in addition to natural rainfall to promote establishment. Trees were grown under these conditions for 14 months prior to their use, and were healthy when sampled. On July 2008, phloem samples were taken from 2-year-old sections of a single branch on each tree. Branches on different trees were matched for the relative position on the tree. A 10 cm long section of each branch was cut, placed immediately in a plastic bag on dry ice, and held at −20° C in the laboratory until extraction and analysis of chemical defenses.

Defense Protein Extraction and Analysis Bark (containing phloem) samples of each species were obtained by dissecting branches with a razor blade. Approximately 1 g of bark (from the periderm to vascular cambium) was stripped from each branch, chopped with a razor blade, and ground in a mortar and pestle with liquid nitrogen. Soluble proteins were extracted from ground tissues in ice-cold 0.01 M sodium phosphate buffer pH 6.8 containing 5% polyvinylpolypyrrolidone (PVPP). Homogenates were centrifuged for 10 min at 7,000×g, and the cleared supernatants were transferred to fresh tubes. Acetone precipitation of soluble proteins was performed on the cleared homogenates. Ten ml of cold acetone were added to 2 ml of extract and vortexed. Tubes were placed at −20° C for 30 min. After centrifugation at 7,000×g for 10 min, the supernatant was removed, and the pellet was air dried for 30 min. Pellets were resuspended in 1.5 ml of 0.01 M sodium phosphate buffer pH 6.8 by vortexing, and extracts were stored at −20° C until they were analyzed. Activities of polyphenol oxidase, peroxidase, and trypsin inhibitor were assessed in soluble protein extracts as in Cipollini et al. (2004). Briefly, polyphenol oxidase and peroxidase activity were assessed using caffeic acid and guaiacol as substrates, respectively. Trypsin inhibitor activity was assessed by examining the inhibition of trypsin in a radial diffusion assay. Transcripts of serine proteases, like trypsin, recently have been found in the gut of EAB larvae (Mittapalli et al., 2010). Phenol oxidizing activities were expressed and statistically analyzed as $\Delta Abs_{470nm} \cdot min^{-1} \cdot$ mg extract protein^{-1} and $\Delta Abs_{470nm} \cdot min^{-1} \cdot$ g fresh mass^{-1} . Trypsin inhibitor activities were expressed and statistically analyzed as mg Tryspin Inhibitor · g extract protein^{-1} and mg Trypsin Inhibitor · g fresh mass^{-1}.

Browning Reaction The browning reaction can be observed within a few minutes around wounded phloem tissues of ash, and is driven in plant tissues primarily by the action of phenoloxidases (including both peroxidase and polyphenol oxidase) on endogenous phenolics resulting in quinone formation and subsequent polymerization (Cheng and Crisosto 1995; Dehon et al. 2002). New extracts were made for the browning rate analysis. The extraction procedure was similar to that for defense protein analysis except that the original extraction buffer did not contain PVPP in order to keep water-soluble phenolics in the extracts, and no precipitation step was performed. The browning reaction was examined immediately after removal of supernatants from the centrifuged phloem homogenates. Aliquots (0.3 mL) of each soluble extract were placed in duplicate in a standard 96-well plate, and the absorbance at 470 nm was recorded twice; once before and once after a 1 hr incubation at room temperature. The rate of the browning reaction was expressed as $\Delta Abs_{470nm} \cdot hr^{-1} \cdot g\ FW^{-1}$.

Total soluble protein in extracts was measured using bovine serum albumin as a standard and Bio-Rad protein dye reagent, as described in Bradford (1976). We used these estimates to calculate total soluble protein of the phloem on a fresh mass basis. All assays were performed in duplicate.

Soluble Phenolic Extraction and Total Phenolic Analysis - Phloem samples of each species were ground in liquid nitrogen, weighed to 100 mg, and extracted twice with 0.5 ml of HPLC grade 100% methanol for 24 h in the dark at 4° C. Extracts were centrifuged for 5 min at 13,400×g and the two supernatants were pooled into ~1 ml of extract. Supernatants were used for soluble phenolic analyses, and the pellet was used for lignin analyses. Total soluble phenolic content was analyzed using the Folin-Ciocalteau method, modified from Bonello and Pearce (1993). In brief, 0.002 ml of methanol extract was added to 0.075 ml of methanol and 0.5 ml of water and mixed, then 0.0375 ml of Folin's Phenol Reagent was added (Sigma, St. Louis, MO, USA). Three minutes later, 0.0375 ml 1 M $NaHCO_3$ was added, and the solution was mixed thoroughly. After a 1 hr incubation at room temperature, the samples were analyzed spectrophotometrically against a standard curve of gallic acid at 725 nm, and total soluble phenolic concentration was expressed as mg phenolics (gallic acid equivalents) per g fresh mass.

Quantification and Identification of Soluble Phenolics with HPLC-UV Analysis of phenolic compounds was performed on an Alliance 2690 separation module (Waters, Milford, MA, USA) equipped with an autosampler, and a 996 Photodiode Array Detector (Waters). The autosampler and column temperatures were set to 4 and 30° C, respectively. The binary mobile phase consisted of water/acetic acid (A) (98:2, *v*/*v*) and methanol/acetic acid (B) (98:2, *v*/*v*), with a flow rate of 1 ml/min. The elution program followed that of Eyles et al. (2007). The injection volume for all samples was 0.010 ml. Samples were passed through a Photodiode Array Detector (PDA) (scanning range, 200–400 nm). Analysis of individual compounds was achieved by combining 0.100 ml from each biological replicate into a pooled sample for a total of 4 sample pools [Manchurian, white, green (wild), and green (cv.)]. Sample pools then were run using the HPLC-UV conditions above, and individual compounds with peak areas exceeding 0.05 absorbance units (AU) were chosen for further analyses in individual samples. Compound identities were based on an exact match to λ_{max} and retention time (RT) data presented in Eyles et al. (2007) and Chen et al. (2011). These sources present compound identities based on congruence of mass spectral information, λ_{max}, and RT with that of the literature, and in some cases generate NMR data in order to provide definitive structural information of individual

unknown compounds. Instrument and gradient parameters used in our analyses were conducted following the methods of Eyles et al. (2007) and Chen et al. (2011) using the same instrument, column, methods, and parameters. The identity of compound 32 (luteolin) was unequivocally confirmed by comparing λ_{max} and retention time (RT) against an external standard. Relative amounts of individual compounds were quantified by peak areas observed at 280 nm.

Lignin Extraction and Analysis Extraction of lignin was accomplished following the method of Bonello et al. (1993), with minor modifications. Pellets left over from extraction of soluble phenolics were washed once each with 1 ml of Milli-Q water and HPLC grade methanol (Fisher). Pellets then were washed with 0.9 ml of tert-butyl methyl ether (Sigma) and allowed to air dry in a fume hood overnight. Pellets were incubated with the addition of 0.4 ml of 1 M NaOH on a shaker at 40 ° C for 21 hr. The reaction mixture was acidified with 0.2 ml of 1.5 M formic acid (Sigma), followed by 0.4 ml of HPLC grade methanol. Pellets were washed with 1 ml of HPLC grade water and resuspended in 0.8 ml of 2 M HCl and 0.3 ml of thioglycolic acid (Sigma). Samples were incubated at 86 ° C for 4 hr, and supernatants were discarded. Pellets were rinsed twice with 1 ml of water. Pellets were resuspended in 1 ml of 0.5 M NaOH, and the tubes were placed on a shaker for 18 hr at 200 rpm twice, and supernatants were pooled from each sequential extraction. The combined solution was acidified with 0.3 ml of concentrated HCl at room temperature for 4 hr, centrifuged for 5 min, and supernatants were discarded. The lignin–thioglycolic acid pellets were air dried in a fume hood and then re-solubilized in 1 ml of 0.5 M NaOH. In all steps, tubes were vortexed after adding solutions, and supernatants and pellets were separated by centrifugation. Lignin was quantified spectrophotometrically by comparing the absorbance of a 40:1 dilution of samples in 0.5 M NaOH at 280 nm against a standard curve of spruce lignin and is expressed as mg lignin per g fresh biomass (Sigma-Aldrich, St Louis, MO, USA).

Statistical Analysis Defense protein activities, total protein concentration, lignin concentration, total phenolic concentration, and rates of the browning reaction were compared among the three ash cultivars and one wild population using one-way ANOVA in SAS (SAS Institute, Cary, NC, USA). Transformations were applied to the data as needed to meet the assumptions of ANOVA. Means were separated using Tukey's tests.

Differences in phenolic profiles among the ash species and cultivars (based on peak areas) were evaluated with Principal Components Analysis (PCA) using the "ade4" package (Version 1.4–14, Dray and Dufour, 2007) in R (Version 2.10.1, R Development Core Team, 2009). We used the methods of Doledec and Chessel (1987) to estimate the contribution of tree identity to inertia decomposition in the model. Briefly, the method estimates the variation between classes (from the centroid of data associated with each class relative to that of all other classes) relative to variation within each class (from the individual data points within a class to the centroid of that class); this between-classes inertia is represented as a percentage of the total inertia in the data. Statistical significance is determined by a Monte-Carlo test, in which the observed between-classes inertia is compared to a distribution of estimates obtained after repeated randomization of the data matrix.

Two analyses were performed. The first included all 33 phenolic compounds, some of which were absent from one or more of the tree species. This analysis was performed on rank-transformed data to account for variance scaling with the concentration of detected compounds and overdispersion resulting from certain compounds not being detected in several samples. Where concentrations were equal among individual trees, observations were ranked in random order; estimates of between-classes inertia were robust in our tests of this ranking method, remaining consistent up to seven places after the decimal. The second analysis included only those compounds that were found in all tree species, although not necessarily in all trees within each species (oleoside/syringin, nunzenhide-related compound, pinoresinol-related compound, oleuropein, and apigenin); data also were rank-transformed prior to this analysis. For each analysis, we estimated contrasts for the specific comparison between the resistant species (Manchurian ash) and the other species. We also estimated contrasts between white and green ash cultivars and between the green ash cultivar and the green ash grown from wild seedlings, based on subsets of the rank-transformed data used in the first analysis.

Results

Peroxidase Activity Peroxidase activity varied among species, both per unit protein ($F_{3,\ 35}$=4.17, P=0.013) and per unit fresh mass ($F_{3,\ 35}$=10.5, P<0.001) (Table 1). Per unit protein, the green ash cultivar and the wild population showed the highest peroxidase activity, white ash was lowest, and Manchurian ash was intermediate. Per unit fresh mass, the green ash cultivar, the wild green ash, and Manchurian ash had similarly high activities, and white ash had lower activity.

Polyphenoloxidase Activity Polyphenoloxidase activity did not vary among species either per unit protein ($F_{3,\ 35}$=2.06, P=0.123), or per unit fresh mass ($F_{3,\ 35}$=1.48, P=0.236).

Table 1 Mean (± SD) levels of several defense traits in phloem of branches from 5 year old ash saplings grown in a common garden. means with different lower case letters are significantly different at α=0.05

Tree identity	POD/mg protein	POD/g FM	PPO/mg protein	PPO/g FM	mg TI/g protein	Total protein	Total phenolics	Browning rate
Manchurian	158.9(104.5)^{ab}	22.0(9.3)^{a}	25.2(19.4)	3.4(2.3)	28.1(8.70)^{b}	0.48(0.16)	7.21(1.8)^{ab}	1.04(0.26)^{a}
Green cv.	266.2(61.3)^{a}	25.9(5.8)^{a}	47.0(17.1)	4.5(1.6)	50.7(25.9)^{a}	0.31(0.12)	9.86(3.0)^{a}	0.86(0.22)^{ab}
Green wild	255.9(105.1)^{a}	24.2(9.5)^{a}	52.8(30.0)	4.7(3.2)	51.4(27.9)^{a}	0.40(0.23)	9.97(3.4)^{a}	0.78(0.27)^{ab}
White	126.3(39.8)^{b}	12.4(2.8)^{b}	29.7(16.6)	2.9(1.5)	46.3(13.6)^{ab}	0.32(0.13)	4.93(3.1)^{b}	0.61(0.09)^{b}

POD Peroxidase Activity, *PPO* Polyphenoloxidase Activity, *TI* Trypsin Inhibitor Activity, *FM* Fresh Mass

Trypsin Inhibitor Activity Trypsin inhibitor varied among species per unit protein ($F_{3,\ 35}$=3.26, P=0.033, Table 1), but not per unit mass ($F_{3,\ 35}$=0.275, P=0.843, data not shown). Per unit protein, the green ash cultivar and the wild green ash had the highest activity, Manchurian ash had the lowest, and white ash was intermediate.

Protein Content There was a tendency for total soluble protein concentration to vary among species ($F_{3,\ 35}$=2.44, P=0.081) (Table 1). In particular, Manchurian ash tended to have higher protein concentrations in the phloem than white and green ash.

Rate of Browning Reaction The browning rate of phloem extracts varied among species ($F_{3,\ 35}$=6.34, P=0.002) (Table 1). Manchurian ash had the fastest browning rate, the green ash cultivar and wild green ash had intermediate rates, and the white ash cultivar had the slowest rate.

Soluble Phenolic Concentration Significant differences in total soluble phenolic concentrations were found among ash species ($F_{3,\ 35}$=6.88, P<0.001, Table 1). Phenolic concentrations in the green ash cultivar and the wild green ash were similar and highest, Manchurian ash was intermediate, and white ash had the lowest level.

Lignin Concentration Lignin concentration averaged 16± 2 mg/g fresh mass and did not vary significantly among species ($F_{3,\ 35}$=0.299, P=0.825).

Phenolic Profiles Phloem phenolic profiles generally were consistent within a species (or cultivar) and distinct between species (or cultivars) with tree identity accounting for 47.8% (all compounds, P<0.001) and 41.8% (common compounds, P<0.001) of inertia decomposition in the PCA. Patterns in the expression of all phenolic compounds suggested a separation between Manchurian ash and the other ash species, with this contrast accounting for 26.0% (all compounds, P<0.001) of inertia decomposition (Fig. 1). Much of this pattern is due to the presence of certain compounds in Manchurian ash that are absent in the other species, and *vice versa*. However, this contrast was also significant (26.3%, P<0.001) when restricting the analysis to five compounds that were expressed in all species (Fig. 2).

Phenolic profiles also indicated differences among the ash species that were due to the concentrations of expressed compounds. Green and white ash cultivars were easily separated, with 20.7% of inertia decomposed between the two species (P<0.001). The green ash cultivar and the wild green ash were also distinguished (P<0.001) but with reduced contribution of cultivar identity (15.7%).

Of the 33 compounds in the analysis, Manchurian ash contained nine compounds not found in the other species, including homovanillic alcohol, esculin, caffeoyl quinic acid, fraxidin hexoside, fraxetin, pinoresinol dihexoside, calceolarioside A, calceolarioside B, and one unknown (Table 2 and Fig. 3). It also had high concentrations of mandshurin and fraxin, which were found at lower levels in some of the other species. Four compounds were found in North American ash species but not in Manchurian ash, including pinoresinol, luteolin, and two unknowns. However, the profile of North

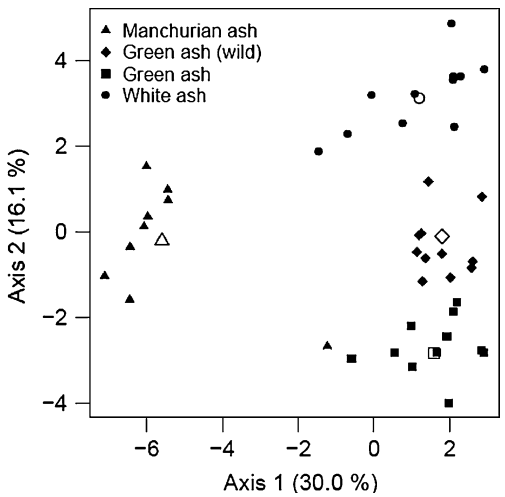


Fig. 1 Principal component analysis of phenolic compounds in phloem extracts from branches of 5-year-old ash trees grown in a common garden. Data were rank-transformed prior to analysis. Closed symbols represent the coordinates of each sample, while open symbols represent the estimated centroid of the points for each of the cultivars. Percentages indicate the inertia decomposition associated with each of the two axes

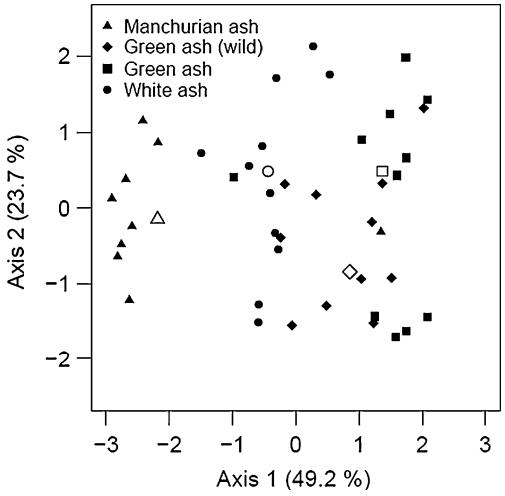


Fig. 2 Principal component analysis using only the phenolic compounds that were detected in all ash cultivars in this study. Compound concentrations were estimated from phloem extracts from branches of 5-year-old ash trees grown in a common garden. Data were rank-transformed prior to analysis. Closed symbols represent the coordinates of each sample, while open symbols represent the estimated centroid of the points for each of the cultivars. Percentages indicate the inertia decomposition associated with each of the two axes

American ash was dominated strongly by syringin/oleoside, which was present in much smaller amounts in Manchurian ash. They shared four other compounds: apigenin, a pinoresinol-related compound, nunzenhide-related compound, and oleuropein, with Manchurian ash having a higher concentration of the pinoresinol-related compound. Verbascoside was found in all but white ash, and hydroxytyrosol hexoside was found in all but the wild green ash. Rutin and a rutin-related compound were found only in white ash. The green ash cultivar and wild population were similar in most instances, but tyrosol, a hydroxytyrosol related compound and one unknown were found only in the cultivar, and verbascoside, verbascoside hexoside, and one unknown were found only in the wild population.

Discussion

Manchurian ash, which shares an evolutionary history with EAB in northeastern Asia, exhibits much higher resistance to attack to this insect than North American ash species (Rebek et al., 2008). The central objective of this experiment was to determine if a suite of constitutive chemical defense traits in the phloem of Manchurian ash, sampled during the growing season phenologically coincident with feeding of EAB larvae, vary qualitatively or quantitatively from susceptible North American ash species in ways that may help to explain this pattern. In particular, we hypothesized that Manchurian ash would exhibit higher levels of selected defense traits and/or possess unique defense attributes. Manchurian ash (cv. Mancana) exhibited such features as higher total soluble protein concentration and a higher rate of the browning reaction in phloem extracts, and contained several soluble phenolic metabolites not found in the North American ash species. In addition, quantitative differences were found in soluble phenolics that Manchurian ash shared with the North American ashes, and it lacked a few compounds that were found in North American species. White ash (cv. Autumn Purple), a highly susceptible cultivar, generally showed the lowest levels of several defense traits, but a susceptible green ash cultivar (cv. Patmore) and green ash plants grown from a wild population did not. While specific resistance mechanisms to EAB in ash are still unknown, constitutive variation in some of these putative defense traits may help to explain variation in EAB attack rates and survival among different ash species.

While the highly resistant Manchurian ash did not exhibit the highest activities of the phenol oxidizing enzymes, total phenolic concentration and trypsin inhibitors, white ash, a highly susceptible species, tended to have low activities of phenol oxidizing enzymes, a slow rate of browning, and a low total protein concentration. White ash generally shared the same phenolic profile as green ash, as expected based on their phylogenetic relatedness (Wallander, 2008), but was clearly separable from both green ash and Manchurian ash based on having reduced quantities of phenolics that all species shared (as well as lacking several compounds produced by Manchurian ash) and lower activities of some defense proteins. Each of these findings is consistent with expectations of a highly susceptible species. However, green ash is also susceptible yet had high activities of phenol oxidizing enzymes, total phenolic concentrations, and relatively high activities of trypsin inhibitors. Since it largely shared a phenolic profile with white ash, either its susceptibility to EAB is explained in part by its lack of some of the unique compounds that Manchurian ash possesses, or the susceptibility of both white and green ash is due to feeding stimulants or other insect growth-promoting traits not possessed by Manchurian ash. In addition, we found that the green ash cultivar (cv. Patmore), and the green ash from the wild population were similar on the basis of nearly all protein, browning, lignin traits measured, but some variation was seen between them on the basis of individual phenolic metabolites. This suggests that findings on field resistance to EAB and resistance mechanisms using this cultivar of green ash should largely represent that found in wild green ash.

While the mechanisms of resistance in ash species to insects are largely unknown, phenolics as a class commonly are implicated in resistance to insects in many plants (Levin, 1976). Our multivariate analysis, based on 33

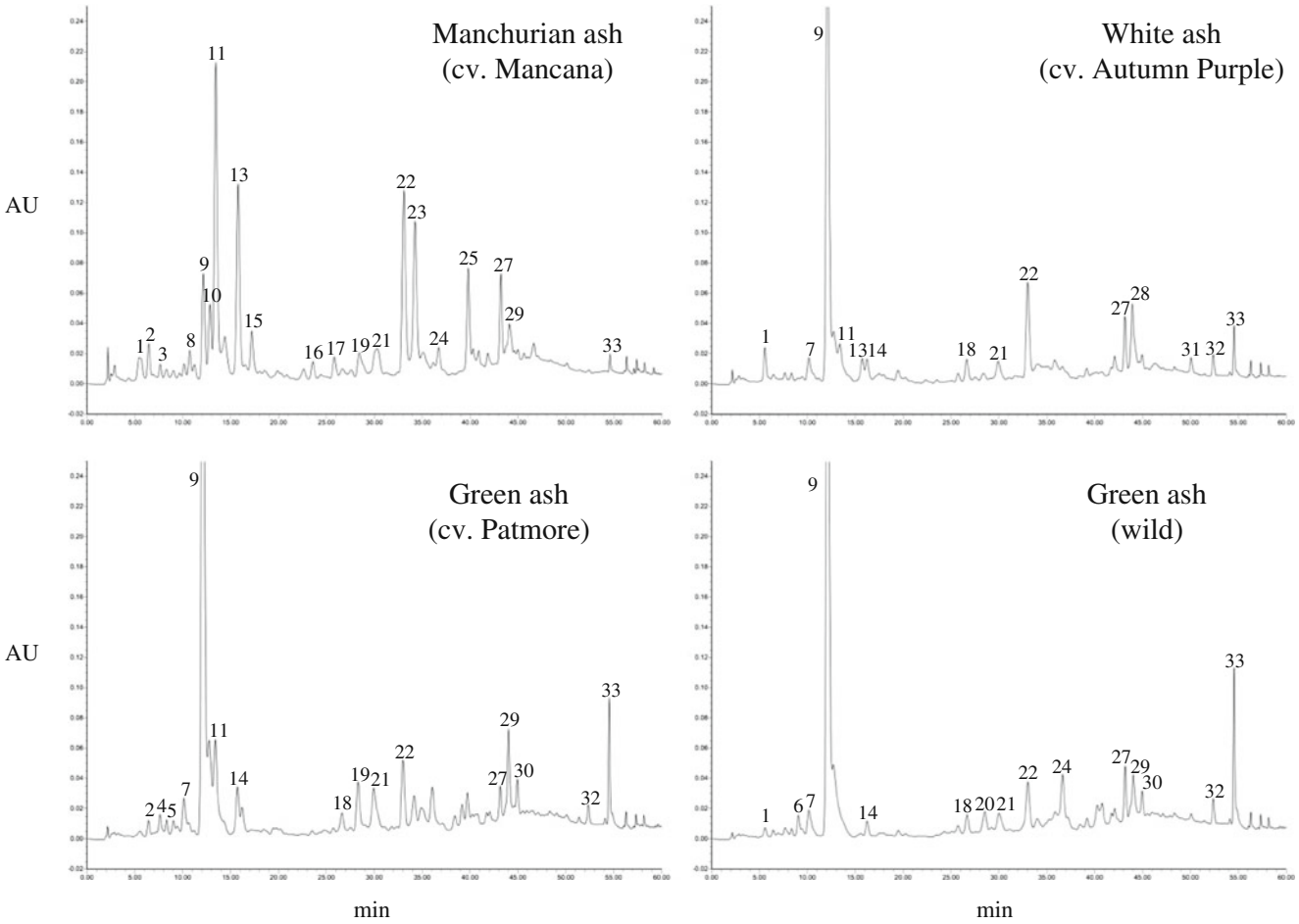


Fig. 3 HPLC chromatograms at 280 nm of pooled phloem extracts of 5-year-old ash trees grown in the common garden. *AU* absorbance units. Peak numbers correspond to Table 2

compounds, showed that Manchurian ash and white ash were both clearly distinguishable from each other, and both were distinguishable from green ash. While the green ash cultivar and wild green ash also differed from each other in multivariate space, this difference was less than the among-species differences. Many of our findings using growing saplings were consistent with findings using bare-root dormant stems (Eyles et al., 2007), but some differences were observed. Manchurian ash had nine compounds not found in the other species, which included several hydroxycoumarins, lignans, and phenylethanoids. These were mostly the same as those identified by Eyles et al. (2007), except that we did not find methylesculin or esculetin in growing stems of Manchurian ash that were found previously in dormant stems. In both studies, the phenolic glycoside syringin dominated the phenolic profile of green and white ash, and was absent or at low levels in Manchurian ash. Since we sampled trees growing in the field during a time period when EAB larvae would be actively feeding, results of the current study may be particularly relevant.

The mechanisms of resistance to EAB in Manchurian ash have not been determined, but some of the phenolic compounds it produces have been examined for their biological effects on insects and pathogens. Hydroxycoumarins like esculin have been suggested by Silva et al. (2006) to retard insect development by inhibiting trehalase, a major enzyme that controls glucose availability in insects. In cassava, some of the same hydrocoumarins as those found in Manchurian ash, were shown by Rodriguez et al. (2000) to have possible anti-microbial activities against a variety of fungi and bacteria.

Manchurian ash had high levels of some lignans, including pinoresinol dihexoside and a pinoresinol related compound. Pinoresinol was found at high levels in dormant stems of Manchurian ash (Eyles et al., 2007), but found only in North American ashes in this study. Lignans have wide biological activities, and effects on insects have been well documented for some of them (Cabral et al., 1999; Garcia et al., 2000; Rios et al., 2002; Schroeder et al., 2006). Other compounds found either exclusively or at higher levels in Manchurian ash, such as homovanillic

Table 2 Characteristics, identity, and mean (± sd) peak areas ($\times 10^{-5}$) of 33 unknown and tentatively identified phenolic compounds in phloem extracts from branches of 5-year old ash trees grown in a common garden

Peak number	Retention time (min)	UV max (nm)	Compound identity	Reference	Manchurian cv. Mancana	Green cv. Patmore	Green wild	White cv. Autumn Purple
1[b]	5.62	277.7	Hydroxytyrosol hexoside	Eyles et al., 2007	4.44 (1.57)	nd	0.848 (0.407)	6.10 (5.49)
2	6.45	276.5	Unknown 1		7.0 (6.41)	2.23 (4.63)	nd	nd
3[b]	7.45	275.3	Homovanillic alcohol	Eyles et al., 2007	2.89 (1.43)	Nd	nd	nd
4[b]	7.61	275.3	Hydroxytyrosol related cmpd	Eyles et al., 2007	nd	3.88 (2.09)	nd	nd
5[b]	8.31	276.5	Tyrosol hexoside	Eyles et al., 2007	nd	2.01 (0.91)	nd	nd
6	9.04	280.1	Unknown 2		nd	nd	3.64 (2.2)	nd
7	10.13	265.1, sh 305	Unknown 3		nd	7.01 (1.74)	4.70 (2.73)	5.32 (4.44)
8[b]	10.71	334.7, sh 290	Esculin	Eyles et al., 2007	5.27 (2.4)	Nd	Nd	nd
9[b]	12.12	264.7	Oleoside/Syringin	Eyles et al., 2007	13.6 (32.1)	136 (48.9)	114 (41.7)	92.9 (5.78)
10[b]	12.85	290.7, 335.9	Caffeoyl-quinic Acid	Eyles et al., 2007	11.6 (4.15)	nd	nd	nd
11[b]	13.42	341.9, sh 300	Fraxin	Eyles et al., 2007	49.4 (25.2)	nd	nd	6.79 (5.89)
12[b]	14.41	290.7, sh 335	Fraxidin Hexoside	Eyles et al., 2007	6.74 (1.41)	nd	nd	nd
13[b]	15.76	320.7	Mandshurin	Eyles et al., 2007	37.3 (14.0)	nd	nd	8.39 (7.45)
14	16.24	315.7	Unknown 4		Nd	4.53 (2.16)	2.60 (1.65)	4.35 (3.67)
15[b]	17.21	337.1	Fraxetin	Eyles et al., 2007	9.40 (2.21)	nd	nd	nd
16[b]	23.58	276.5	Pinoresinol Dihexoside	Eyles et al., 2007	3.62 (1.37)	nd	nd	nd
17	25.81	314.5, 339.5	Unknown 5		3.71 (2.99)	nd	nd	nd
18[b]	26.65	277.7	Pinoresinol	Eyles et al., 2007	Nd	4.17 (0.79)	3.33 (2.34)	4.52 (2.62)
19[b]	28.35	278.9	Pinoresinol Related Cpd. 1	Eyles et al., 2007	1.12 (0.66)	1.92 (1.85)	nd	nd
20[b]	28.54	332.3, sh 285	Verbascoside Hexoside	Eyles et al., 2007	nd	nd	7.70 (6.02)	nd
21[b]	30	278.9, 331.1	Nunzenhide Related Cpd	Chen et al., 2011	5.31 (3.63)	9.85 (5.99)	4.50 (3.73)	6.43 (3.25)
22[b]	33.02	278.9	Pinoresinol Related Cpd 2	Eyles et al., 2007	35.1 (17.7)	10.8 (5.74)	8.25 (4.74)	19.3 (8.7)
23[b]	34.25	328.7, sh 290	Calceolarioside A	Eyles et al., 2007	35.2 (45.3)	nd	nd	nd
24[b]	36.68	331.1, sh 290	Verbascoside	Eyles et al., 2007	8.65 (6.3)	nd	15.9 (14.2)	nd
25[b]	39.80	328.7, sh 290	Calceolarioside B	Eyles et al., 2007	28.3 (33.6)	nd	nd	nd
26[b]	42.10	259.9, 350.2	Rutin Related Cmpd	Chen et al., 2011	nd	nd	nd	4.04 (1.99)
27[b]	43.18	280.1	Oleuropein	Eyles et al., 2007	10.3 (7.41)	2.96 (0.84)	6.81 (3.91)	6.16 (5.03)
28[b]	43.902	257.6, sh 295, 357.4	Rutin	Chen et al., 2011	nd	nd	nd	12.7 (5.96)
29[b]	44.04	sh 280	Ligustroside	Eyles et al., 2007	8.74 (10.1)	15.4 (9.76)	8.69 (7.69)	nd
30[b]	44.96	265.9, 337.1	Apigenin-7-*O*-rutinoside	Chen et al., 2011	nd	5.61 (2.65)	4.21 (4.22)	nd
31	50.03	290.7, sh 335	Unknown 6		nd	nd	nd	2.21 (1.40)
32[a]	52.39	255, sh 295, 351.4	Luteolin	Matched to Standard	nd	2.73 (1.36)	2.93 (2.23)	2.52 (1.65)
33[b]	54.55	268.2, 339.5	Apigenin	Eyles et al., 2007	3.18 (4.76)	10.3 (4.49)	12.3 (7.50)	4.29 (2.13)

ND Not Detected, *SH* Shoulder

[a] Identity confirmed with authentic standard

[b] Compound identities were based on an exact match to λ_{max} and retention time (RT) data presented in Eyles et al. (2007) and Chen et al. (2011). These sources present compound identities based on congruence of mass spectral information, λ_{max}, and RT with that of the literature, and in some cases generate NMR data in order to provide definitive structural information of individual unknown compounds. Instrument and gradient parameters used in our analyses were conducted following the methods of Eyles et al. (2007) and Chen et al. (2011) using the same instrument, column, methods, and parameters

alcohol, fraxin, fraxidin hexoside, mandshurin, and calceolariosides A and B have not been examined for their bioactivity against insects, but several of them have shown anti-oxidant, anti-cancer, and other medically relevant biological activities (e.g., Deiana et al., 2008). Syringin, which was present at high levels in white and green ash and absent in Manchurian ash, has shown antifeedant effects against some beetle species (Cis et al., 2006), but does not appear to confer resistance to EAB given the susceptibility of these two species. It is possible that some compounds found exclusively in the North American ashes serve as feeding stimulants or growth promoting traits for EAB larvae.

The elevated wound browning rate of Manchurian ash is driven presumably by the action of phenol oxidizing enzymes on water-soluble phenolics present in the extracts. As such, this measure integrates both phenol oxidizing enzyme activity and phenolic content. While Manchurian

ash did not show increased phenol oxidizing enzyme activities in this study, the unique phenolic content and/or increased concentrations of certain phenolics in phloem extracts of Manchurian ash may have contributed to its elevated browning rate. For example, metabolism of some hydroxycoumarins by peroxidase contributed to the browning reaction in cassava root (Buschmann et al., 2000). While this trait has been associated with anti-microbial effects in structures such as fruits (Cheng and Crisosto 1995), it has been assessed infrequently in the context of insect resistance, especially towards wood-boring insects. Dowd and Norton (1995) showed that several simple phenolics in corn callus tissues could be oxidized (browned) through the action of endogenous peroxidases, and browned tissues were poor quality food for the corn earworm, *Helicoverpa zea*. Since the oxidation status of phenolics can determine their toxicity to insects (Appel, 1993), the rapid wound browning of Manchurian ash phloem may contribute to its resistance to EAB if it is an indication of the oxidation status of its particular phenolic mixture.

Resistance to wood boring insects in deciduous trees is little understood (Dunn et al., 1990), and little work has been done specifically on chemical mediation of ash resistance to EAB larval feeding. The results presented here, along with those of Eyles et al. (2007), suggest that clear differences in the chemical defense profile exist between a resistant ash species and its susceptible counterparts in North America. These characteristics may represent defined molecular and biochemical targets for conventional or molecular breeding of ash species, and suggest that the examination of resistant Asian ash species will facilitate the discovery of QTLs or particular resistance genes linked to expression of biochemical and physical defenses. Conventional hybrids between Asian and North American ashes exist (J. Koch, personal communication), but the possibility of enhancing EAB resistance of North American ashes will rely on their ability to appropriately express biosynthetic machinery necessary to express resistant traits, whether it is transferred through traditional breeding efforts, or through genetic transformation. Although we focused on constitutive phloem defenses in this paper, feeding- or wound-induced changes in chemical defense profiles also may be important for resistance to EAB and are the subject of current investigation. We are also examining the direct effects of putative resistance traits on EAB in artificial diets, and further characterizing the browning reaction and its role in resistance.

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Boron Fertilization Enhances the Induced Defense of Silver Birch

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Abstract Boron (B) deficiency is a common micronutrient deficiency that has been reported to affect the phenolic metabolism of plants. Thus, it may play a role in defense against herbivorous animals. However, the role of B in a plant's resistance to herbivores has not received any particular attention from researchers. In this study, we tested the effects of B nutrition 1) on the biochemical and mechanical defenses of birches and the growth of seedlings, and 2) the resistance of seedlings to larvae of the autumnal moth, *Epirrita autumnata*. Boron fertilization improved the resistance of birch, which was shown as reduced pupal weight of the herbivore. However, B fertilized trees suffered from heavier defoliation than unfertilized ones due to compensation feeding of larvae. The growth of the seedlings was diminished, and several biochemical changes occurred in leaves of herbivore seedlings, and B also played a role in these changes. Polyphenoloxidases (PPOs) and peroxidases (PODs) and their substrates, chlorogenic acids, were induced by herbivory in B fertilized seedlings but not in unfertilized seedlings. The lower pupal weights and increased consumption of the herbivores were probably linked to the plants' phenoloxidase-mediated production of reactive quinones, which decrease the nutritive value. Herbivory upon new stems led to an increase in the number of resin glands that provide defense against mammalian herbivores. Herbivory also had a substantially negative effect on B concentration in leaves of B fertilized seedlings. We postulate that B nutrition of trees may play a significant role in the induced defense of birches.

Key Words Birch · Boron · Defense · *Epirrita autumnata* · Herbivory · Immune function · Phenols · Resistance

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Introduction

Boron deficiency is said to be the world's most common micronutrient deficiency among forest trees (Shorrocks, 1997). In Finland, trees suffering from B deficiency often are growing in nutrient rich soils such as in groves, reforested fields, and pastures, or in drained peatlands that have been fertilized with macronutrients (Saarsalmi and Tamminen, 2005). In conifers, B deficiency causes leader dieback and disturbs apical dominance (Hopmans and Clerehan, 1991). The crown of a B deficient tree may be bushy and show several main shoots (Braekke, 1979). In deciduous trees, morphological symptoms of B deficiency are not always as clear as those for conifers.

Physiologically, B deficiency causes several impairments such as cessation of root elongation and growth of apical meristems. Boron plays a role in the establishment of the primary cell wall and the integrity of the plasma membrane. Boron cross-links two pectic polysaccharide rhamnogalacturonan II (RGII) monomers with a borate bridge, thus stabilizing the cell-wall matrix (O'Neill et al., 2004). Interestingly, B deficiency causes effects on phenol metabolism similar to those of herbivory. Both stresses trigger the activity of polyphenol oxidases (PPO) and the accumulation of quinones (Cakmak and Römheld, 1997; Ruuhola et

al., 2008; Anttila et al., 2010). Additionally, the level of reduced ascorbic acid decreases in response to B deficiency (Lukaszewski and Blevins, 1996) and herbivory (Bi and Felton, 1995), which also contribute to the enhancement of quinone formation and reactive oxygen species (ROS) production.

Earlier studies have shown that induced birch PPO activities may explain the rapidly induced resistance (RIR) of mountain birch (*Betula pubescens* sp. *czerepanovii*) to its major defoliator, *Epirrita autumnata* Borkhausen, autumnal moth (Ruuhola et al., 2008; Anttila et al., 2010). Both quinones and ROS are harmful to plant eating animals, decreasing the assimilation of nutrients and producing, for example, mid gut lesions (Bi and Felton, 1995). Foliar PPO activity (Ruuhola et al., 2008) and phenolic concentrations (Ruuhola et al., 2007) correlated negatively with the growth of autumnal moth larvae on previously wounded trees, but had no negative association on unwounded control trees.

Boron may improve the innate immunity of vertebrate animals (Ardó et al., 2008), but there have been no studies conducted with insects. The immunity of insects resembles the innate immunity of vertebrates comprising physical barriers, hemocytes, and induced synthesis of antibacterial compounds (Gillespie et al., 1997). Insects are defended against parasites and diseases with a process called encapsulation, in which an intruder is capsuled by a cell layer. In melanotic encapsulation, this layer is hardened by melanin (Gillespie et al., 1997). Both the cellular and humoral immune systems are involved in the encapsulation process, which can be considered a general measure of the activity of the immune system (Ratcliffe, 1993). It has been demonstrated on Lepidoptera that the ability to encapsulate a foreign antigen correlates with resistance to entomopathogenic fungal disease (Rantala and Roff, 2007), and is associated also with resistance to parasitoids (Smilanich et al., 2009). In earlier studies, we showed that the encapsulation response of *E. autumnata* pupae was strengthened by a mild stress, such as the priming of the host plant (Kapari et al., 2006) or by moderate heavy-metal pollution (van Ooik et al., 2007). However, this response was mitigated by severe stress, such as starvation (Yang et al., 2007) or exposure to acid rain (Ruuhola et al., 2009). Host plant species and quality also affect the encapsulation response of *E. autumnata* (Klemola et al., 2007; Yang et al., 2008).

No previous studies were found that examine the effects of B fertilization on the herbivore resistance of trees. We expected that both a B deficiency and the correction of B deficiency through fertilization might affect herbivore resistance. Boron deficiency could increase resistance *via* an enhanced production and oxidation of phenols. However, fertilization with B has been shown to improve plants' resistance to pathogens (Dordas, 2008), and optimal B nutrition improved the resistance of soybean to herbivores (Beanland et al., 2003). According to the mineral balance hypothesis, optimal balance among minerals enhances plants growth and improves their herbivore resistance (Phelan et al., 1996).

To test these two alternative hypotheses that either boron deficiency or boron fertilization enhances the resistance of trees, we fertilized silver birch seedlings (*Betula pendula* Roth) with a complete nutrient solution either containing an optimal B level, in relation to other nutrients, or without B (See Table 1). We studied the performance of autumnal moth (*E. autumnata*) larvae that fed on these seedlings and the combined effects of the B and herbivory treatments on the biochemical and mechanical defenses and the growth of the seedlings.

Methods and Materials

Study Species Autumnal moth, *E. autumnata*, is a well-studied species. A characteristic feature of this species is its massive outbreaks in northernmost Scandinavia at 10–11 year intervals (Haukioja et al., 1988; Ruohomäki et al.,

Table 1 Time schedule of the experiment and fertilization treatments

Date	Event	N (mg l^{-1})	H_3BO_3 (mg l^{-1})		Cumulative amount of H_3BO_3 (mg)[a]	
			−B	+B	−B	+B
2/9/2009	Planting	0	0	0	0	0
2/20/2009	Larvae on trees	25	0	0.286	0	0
2/27/2009	Raise of the fertilization	50	0	0.572	0	0.086
3/10/2009	All larvae pupated	50	0	0.572	0	0.372
3/25/2009	Collection of chemistry leaves	50	0	0.572	0	0.715
3/31/2009	Completion (dry weights)	50	0	0.572	0	0.715

[a] Seedlings were fertilized three times per week with 100 ml of the complete nutrient solution

2000). Larvae hatch early in spring in synchrony with the bud burst of mountain birch, *B. pubescens* ssp. *czerepanovii* (Kaitaniemi et al., 1997).

Silver or European white birch (*B. pendula*) is widely distributed throughout Eurasia (Hämet-Ahti et al., 1998). Silver birch is a host for a wide variety of insect and mammalian herbivores, and its resistance to these herbivores has been intensively studied (e.g., Rousi et al., 1991; Mutikainen et al., 2000; Huttunen et al., 2008).

Study Design The study was conducted from February to March in 2009, within a greenhouse located at the Botanical Gardens of the University of Joensuu, Finland. We used 1-year-old seedlings of *B. pendula* obtained from a commercial nursery (Finforelia) and a laboratory stock of autumnal moth larvae that were descendants of insects collected as larvae from northernmost Scandinavia. Winter dormant seedlings in their intact peat plug were transferred to 2 l pots filled with quartz sand (particle size 1.5 mm). The day temperature was raised gradually to a maximum range of +22–24°C, whereas the night temperature was set to a minimum of +13°C. The light:dark -cycle was set to 20:4 h, to mimic the light conditions of spring at the same latitude (N 62° 36.059′ E 029° 45.813′). Seedlings were placed into eight large cages (70×120×125 cm) that were divided into two crosswise parts separated by a voile fabric wall (Anttila et al., 2010). The wooden framed sides of the cages were covered with light permeable voile fabric, and the tops with a polyethene filter that transmitted photosynthetically active radiation (PAR). During the experiment, seedlings were fertilized three times per week with 100 ml of a complete nutrient solution (Aphalo and Lehto, 1997), either with an optimal level of B (+B) or a B level of zero (−B) (Table 1). When necessary, seedlings were watered with tap water. Fertilization treatments started 1 week after the potting of the seedlings (Table 1). Newly hatched neonate larvae were allocated to the seedlings synchronized with the bud burst, which was 11 day after potting.

The study setup was factorial: Seedlings were randomly allocated to two treatments with four combinations: no B supply with intact control (–B–H), no B supply with larval treatment (–B+H), B supply with intact control (+B–H), and B supply with larval treatment (+B+H). Six birch seedlings were placed in each half of a cage so that branches of one seedling did not touch another, to prevent the larvae from roaming between seedlings. One half of each cage served as an unwounded control, while the other had six newly hatched larvae released on each seedling at the time of bud burst (altogether 36 larvae per cage). Four of the cages were allocated to the B deficiency treatment (–B), and the other four received the B fertilization treatment (+B).

Larval Performance Larvae were allowed to feed on birch seedlings until they reached the final stage (fifth instar) when they prepared to pupate (3 week). Shortened larvae were collected onto moist unfertilized peat in 48 ml plastic vials where they were allowed to pupate. After 7 day, pupae were weighed, and their sex was determined. Their encapsulation rate was measured by inserting a 2 mm long sterile nylon filament into the pupa (Kapari et al., 2006). The encapsulation reaction was allowed to continue at +23°C for 1 h, after which the filament was removed. Then, the pupae were flash frozen in liquid nitrogen and stored at −80°C prior to analysis. Later, the implanted portions of the filament were photographed from two different angles under a light microscope with a digital camera. These images were analyzed using the image processing software, Image J program. The degree of melanotic encapsulation was analyzed as grey values of reflected light from the implants. As a measure of the encapsulation response, the average grey values from two digital images were used. The data were transformed so that the darkest grey values corresponded to the highest encapsulation response. The transformation was carried out by subtracting the observed grey values from the control grey value (clear filament) (see Rantala and Kortet, 2003).

Sampling of Seedlings When all larvae had pupated, qualitative visual assessments of the seedlings' defoliation percentages were made. Seedlings were allowed to grow and develop new shoots and leaves for a further 2 week, after which they were harvested to measure their defenses (Table 1). Leaves used for enzymatic assays were collected from long shoots: the young (3–4th node) leaves were carefully removed with their petiole, then immediately frozen in liquid nitrogen and stored at −80°C prior to analyses. Leaves for chemical analyses were collected from the main shoots from the 2nd to 4th node; these leaves were dried at room temperature (RT) for 1 week after which leaves were stored at −20°C prior to extraction and high-pressure liquid chromatography (HPLC)-analyses. Phenolic and enzymatic analyses were made for two seedlings from each half of a cage (eight seedlings/treatment). The other four seedlings per cage were used for inductively coupled plasma (ICP)-analysis of the B concentration (16 seedlings/treatment).

The number of new shoots and the number of resin glands per cm^2 on the stems of new main shoots were counted 3 week after larval pupation (Table 1). Since the length of new stems differed greatly between the herbivore and control treatment (from 0.5 to 10 cm), the number of glands in the middle of the new shoot was counted over a 2 mm^2 area. This was later converted to the number of resin glands/cm^2. There appeared to be a clear difference between the control and the herbivore-treated seedlings with respect to the hairiness of their new shoots. To test this

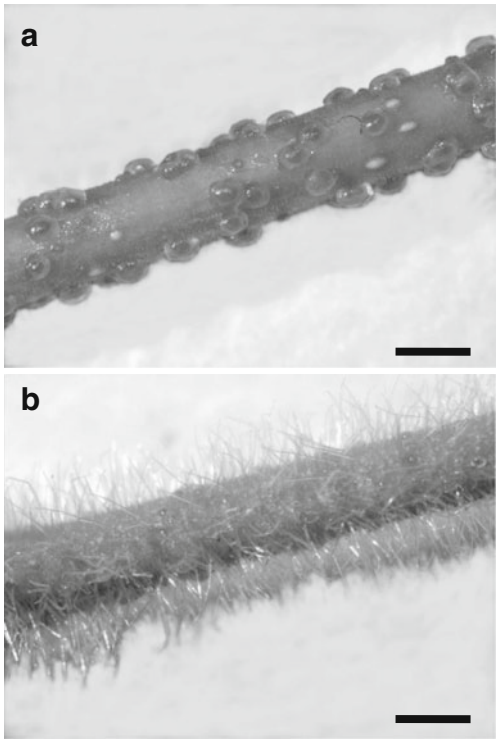


Fig. 1 Stems of *Betula pendula* control seedlings (**a**) and herbivore-treated seedlings after the defoliation by *Epirrita autumnata* larvae (**b**). Differences in stem hairiness between the control and herbivore-treated seedlings were visually grouped to six qualitative classes: first class is a bald stem without hairs (**a**) and the sixth class is a stem covered with long and dense hairs (**b**)

difference we created a qualitative visual pilosity classification system with a range of from 1 to 6 according to the length and density of the hairs on the stem: 1 = bald without hairs, 2 = nearly bald, 3 = dense downy hairs, 4 = dense with thicker and longer hairs, 5 = denser with longer hairs, and 6 = densest with longest hairs (Fig. 1).

The heights of seedlings were measured prior to any treatments (Time 0; Fig. 4) and twice during the experiment: when all shortened larvae had been removed from the seedlings (Time 1; Fig. 4) and after a 2-week-recovery period (Time 2; Fig. 4). The stem biomass was determined by cutting the stems into two pieces, the current and previous season's stems, then drying these parts at +105°C for 3 day, before they were weighed.

Chemical Analyses For the phenolic compound analysis, leaf samples were dried at room temperature for 1 week, then 3–4 leaf disks, without any midrib, were cut from them. Leaf disks (3–4 mg) were homogenized for 30 s, at 5,500 rpm in 600 µl of 100% (v/v) methanol using a Precellys® 24 homogenizer (Bertin Technologies, France). Homogenized samples were kept in an ice bath for 15 min and centrifuged at 13 000 rpm for 5 min at +4°C (Eppendorf® centrifuge 5415 R, USA). The supernatant was taken to a 6 ml tube; extraction was repeated × 3, and with standing on ice for 5 min between extractions. The methanol from the combined extracts (2.4 ml) was evaporated to dryness in the vacuum centrifuge (Eppendorf® concentrator, Germany). Dried samples were redissolved in a solvent of 300 µl of 100% methanol and 300 µl of dH_2O. Dissolved samples were analyzed with Agilent's Series1100 high-pressure liquid chromatography (HPLC) system (Agilent Technologies, Germany) equipped with Agilent's G1315B diode array detector (DAD), and a reversed-phase (RP) octadecyl carbon chain (C18) column (Agilent Technologies, USA) was used for the separation of the compounds as described by Julkunen-Tiitto and Sorsa (2001). Compounds were quantified at 320 nm based on their UV-spectra and retention times, except (+)-catechin and 3,4′-dihydroxypropiophenone-3-β-D-glucopyranoside (DHPPG), which were quantified at 220 and 270 nm, respectively. Concentrations were calculated with the following standards: kaempferol 3-rhamnoside for kaempferol 3-rhamnoside, apigenin 7-glucoside for apigenin derivatives, hyperin for quercetin derivatives, myricetin 3-rhamnoside for myricetin derivatives, (+)-catechin for catechin, chlorogenic acid for chlorogenic acid derivatives, and cinnamic acid for cinnamic acid derivatives. Condensed tannins (CTs) were determined with an acid-butanol test (Porter et al., 1986) on both the soluble methanol fraction (soluble CTs) and the extraction remnants (cell wall-bound CTs), with quantification based on a purified *B. nana* leaf tannin.

Proteins were extracted similarly to the procedure described in Ruuhola and Yang, (2006), except that a raw extract, without purification through desalting columns, was used. The protein extracts were stored at −80°C prior to enzymatic assays. Polyphenol oxidase (PPO) activity was measured with 20 mM catechol as a substrate in 0.1 M K-phosphate buffer, pH 6.4, and the rate of change at 420 nm was followed at room temperature for 5 min with a spectrophotometer (Cary 50 bio UV-VIS spectrophotometer; Varian, Mulgrave, Australia). Peroxidase (POD) activity was measured with 3 mM guaiacol and 0.25 mM H_2O_2 as substrates in 0.1 M K-phosphate buffer, pH 5.8, and the rate of change at 470 nm was followed at room temperature for 5 min. Protein contents of the enzyme extracts were determined with a Bichinchonic Acid Protein Assay Kit (Sigma) based on the Lowry et al. (1951) method. Enzyme activities were expressed as U mg $protein^{-1}$ or U g fw^{-1}: one unit being the amount of enzyme required to increase the absorbance by 0.001 min^{-1}.

Boron concentrations of leaves were determined with an inductively coupled plasma atomic emission spectrometer (ICP-OES, Iris Intrepid, Thermo Elemental, Franklin, MA, USA) after microwave wet digestion in HNO_3 (suprapur, Merck) plus H_2O_2 with a method based on SFS-EN ISO 17025 and Epa 3051.

Statistical Analyses Mixed model analysis (SPSS 17.0.) was used on the data. Normal distribution of the residuals was checked with the Kolmogorov-Smirnov test and visually, with predicted values vs. residuals and Q-Q-plot. The effects of the B treatment on the pupal weight, development time, and encapsulation rate of *E. autumnata* were tested with the following model: B treatment, sex, and their interaction were fixed factors, and a tree was nested within a cage as a random factor. To study the effects of treatments in more detail, pair-wise contrasts were tested with LSD-method.

The mixed model analysis also was used to test the chemistry of leaves. The model in this case had the B and herbivore treatments as fixed factors, and the cage was set as a random factor. For the analysis of the stem weights, number of lateral shoots, and mechanical defenses, the initial height of a seedling was set as a covariate in the model to diminish the possible differences in these initial sizes. The height growth of seedlings was tested with repeated mixed model analysis (SPSS 17.0). In this model, time, B treatment, and herbivore treatment were set as fixed factors; time was a repeated item, the tree was a subject, and the cage was a random factor; the initial height of a seedling was a covariate. Pair-wise contrasts were tested with the LSD-method. The effect of the B fertilization on the defoliation percentage of the herbivore-treated trees was tested with Kruskal-Wallis non-parametric test (SPSS 17.0). The correlations between percentage defoliation and growth of seedlings were analyzed with the Spearman's correlation test.

Results

Boron Concentrations The concentration of B in the birch leaves increased markedly (ca. 50%) and significantly ($F_{6.2}$= 26.48; P=0.002) with B fertilization (Fig. 2). However, the B concentration substantially decreased (27%) with herbivory in the +B seedlings ($F_{48.4}$=7.528; P=0.008) but was not significantly affected in the –B seedlings (Fig. 2).

Performance of Autumnal Moth Pupal weights of the *E. autumnata* females grown on the +B seedlings were significantly ($F_{36.80}$=4.515; P=0.040) lower than those of grown on the –B seedlings, while there was no difference in the male pupal weights for these treatments (Fig. 3a). The sex of a moth had no effect on pupal weight ($F_{205.9}$=0.033; P=0.855); the B treatment × sex interaction also was non-significant ($F_{205.9}$=2.574; P=0.110). Development time for females was longer than that for males ($F_{204.5}$=21.99; P<0.001; Fig. 3b), but the B treatment did not affect development rates of either sex ($F_{36.63}$=2.107; P=0.155),

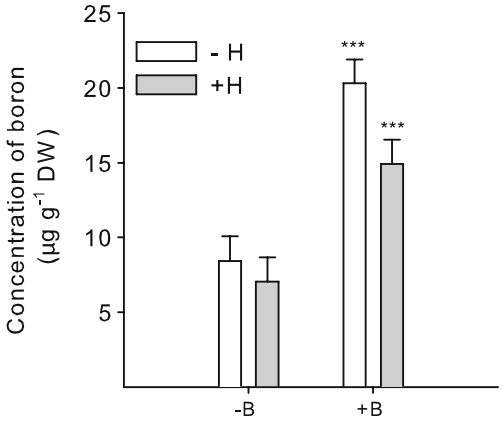


Fig. 2 The boron concentrations (µg g^{-1} DW) in the leaves of *Betula pendula* seedlings exposed to two treatments, boron and herbivory, and their four combinations (−H=unwounded control, +H =herbivore-treated, –B=B unfertilized, +B=B fertilized seedlings). Significant differences (LSD contrasts) between control and herbivory treatments are marked with asterisks (*P≤0.05, **P≤0.01, ***P≤0.001). Data show ESmeans+S.E. (Mixed model analyses, SPSS 17.00). N=16 per treatment

and there was no significant B treatment × sex interaction ($F_{204.5}$=1.774; P=0.194). The encapsulation rate of the pupae tended to be lower in +B treatment than in –B treatment ($F_{33.13}$=2.796; P=0.104; Fig. 3c). Females had a higher encapsulation rate than males ($F_{212.9}$=26.21; P<0.001), but again there was no significant B treatment × sex interaction ($F_{212.9}$=0.161; P=0.689).

Defense of Silver Birch Seedlings Herbivory significantly affected the concentrations of most of the measured phenolic compounds, either by increasing or decreasing their levels, but B fertilization alone had no significant effect. However, there were two significant and three close to significant B treatment × herbivory interactions (Table 2). Quantities of 14 of 26 individual phenolic compounds and the total quercetins, apigenins, and HPLC phenolics increased as a response to herbivory whereas amounts of five compounds decreased with herbivory (Tables 2 and 3). The effect of herbivory on flavonoids was intriguing: myricetin and quercetin 3-arabinosides decreased in the herbivory exposed seedlings, whereas levels of myricetin, quercetin, and kaempferol 3-rhamnosides increased, respectively (Table 3). Equally intriguing was the decrease in soluble condensed tannins (CTs) and total CTs (but not cell wall bound CTs) in the herbivory wounded seedlings. This decrease, however, was significant only for the +B seedlings (Table 3).

More detailed examination of the contrasts (LSD) revealed that herbivory effects were not usually significant for both treatments (Table 3): quantities of 16 compounds were affected in the +B seedlings (e.g., chlorogenic acids and soluble CTs), seven in the –B seedlings (e.g., apigenins

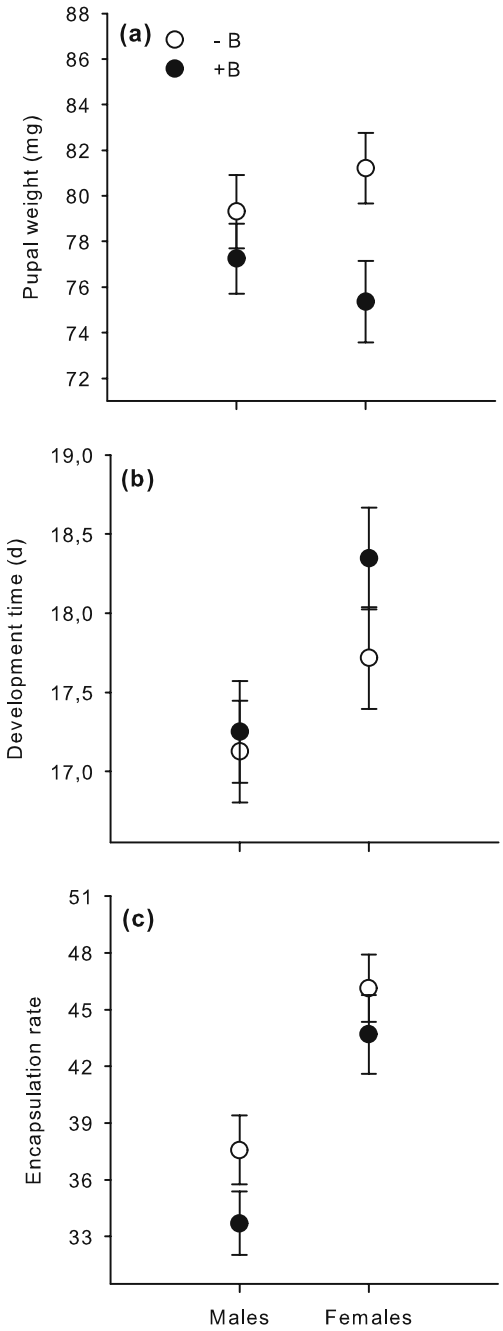


Fig. 3 Pupal weights (**a**), development times (**b**) and encapsulation rates (**c**) of *Epirrita autumnata* males and females grown either on boron unfertilized (−B) or boron fertilized (+B) birch seedlings. Data show ESmeans+S.E (Mixed model analyses, SPSS 17.00). N=112 (−B) and 109 (+B)

and (+)-catechin), and seven were affected in both B treatments (e.g., DHPPG, total cinnamic acids, quercetins, and HPLC phenolics) (Table 3).

Herbivory also seemed to induce the activities of polyphenol oxidases (PPOs) and peroxidases (PODs) as measured both per fresh weight and protein content (Table 2). However, this induction was significant only for the +B seedlings, not for –B seedlings (Table 3). Herbivory exposed seedlings fertilized with B showed a significantly higher POD activity than herbivory seedlings that did not receive B (Table 3).

The number of resin droplets and the hairiness of the stems of new shoots were significantly increased by herbivory (Tables 2 and 3; Fig. 1). There were no significant effects on either of these traits due to the B treatment or a B treatment × herbivory interaction.

Growth of Silver Birch Seedlings The height growth of *B. pendula* seedlings was significantly decreased by herbivory, and there was also a significant B treatment × time interaction, and a nearly significant B treatment × herbivory interaction (Table 4). The growth of +B unwounded control plants tended to be higher than the growth of –B control plants, whereas, the growth of +B seedlings was decreased more than the growth of –B seedlings by the herbivory treatment due to a higher defoliation percentage of +B seedlings (Fig. 4). Boron fertilization led to a higher defoliation percentage (89±4.21%) of the herbivory treatment seedlings ($X^2_1 = 5,904$; P=0.015) than no fertilization (78±4.58%).

Biomasses of the old and new stems and branches also were decreased by herbivory (Tables 5 and 6), indicating that both the growth in height and in diameter was depressed. In contrast, B treatment had no effect on the biomass of stems, and the interaction with the herbivore treatment was not significant either (Tables 5 or 6). The defoliation percentage correlated negatively with the weight of the new (r=−0.675, P=0.004) and old stems (r=−0.704, P=0.002) (data not shown). The number of new lateral shoots increased with herbivory (Tables 5 and 6), apparently due to the loss of apical dominance. The most heavily defoliated seedlings had no clear main shoot and had a bushy appearance at the top. The boron treatment had no effect on the number of lateral shoots (Table 5). However, the effect of herbivory on the number of lateral shoots was significant, but only for the –B seedlings, not for +B seedlings (Table 6).

Discussion

The resistance of silver birch seedlings to autumnal moth was affected by boron (B) fertilization: pupal weights of female moths grown on the B fertilized seedlings were significantly reduced compared to pupal weights of females grown on seedlings that received no B in their nutrient solution. In addition, the immunity of moths, measured by their encapsulation rate, tended to be lower for those that fed on +B seedlings than for those that fed on –B seedlings; however, the moths' development rates were not affected. A lower body weight of a female moth decreases its fitness,

Table 2 The effects of boron and herbivore treatments on the chemical and mechanical defenses of *Betula pendula* seedlings

Defense	B treatment		Herbivory		B treatment x Herbivory	
	F	*P*	*F*	*P*	*F*	*P*
Phenolics (mg g^{-1} dw)						
DHPPG	1.527	0.263	79.14	**<0.001**	4.720	**0.041**
(+)- Catechin	0.002	0.964	5.478	**0.029**	0.899	0.354
Cinnamic acid derivative 1	1.039	0.347	21.40	**<0.001**	2.060	0.165
p-Cinnamic acid derivative 1	0.418	0.542	9.094	**0.006**	1.037	0.319
p-Cinnamic acid derivative 2	0.260	0.630	2.615	0.121	0.155	0.697
p-Cinnamic acid derivative 3	0.098	0.764	0.027	0.871	8.897	**0.011**
p-Cinnamic acid derivative 4	0.010	0.922	16.76	**<0.001**	0.713	0.406
p-Cinnamic acid glycoside	0.644	0.429	10.90	**0.003**	0.586	0.450
Total cinnamic acids	0.230	0.649	19.66	**<0.001**	0.323	0.576
Chlorogenic acid	0.182	0.684	18.77	**<0.001**	2.731	0.113
Neochlorogenic acid	0.011	0.916	9.446	**0.005**	0.460	0.503
Coumaroyl astralgin	0.011	0.921	5.561	**0.028**	0.494	0.490
Myricetin 3-galactoside	0.333	0.585	5.177	**0.033**	0.435	0.516
Myricetin 3-glucoside+-glucuronide	0.070	0.793	10.70	**0.003**	2.892	***0.100***
Myricetin 3-arabinoside	0.686	0.439	3.876	***0.062***	2.279	0.145
Myricetin 3-rhamnoside	3.064	***0.091***	5.505	**0.026**	0.015	0.903
Total myricetins	1.970	0.171	0.953	0.337	0.188	0.668
Hyperin	2.464	0.128	7.830	**0.009**	0.069	0.795
Quercetin 3-glucoside+-glucuronide	0.076	0.785	0.857	0.362	0.164	0.689
Quercetin 3-arabinoside	1.868	0.183	5.243	**0.030**	1.139	0.295
Quercetin rhamnoside	0.418	0.542	26.48	**<0.001**	0.004	0.949
Quercetin derivative	2.091	0.198	7.455	**0.012**	0.748	0.396
Total quercetins	0.000	1.000	16.62	**0.001**	0.069	0.795
Kaempferol 3-rhamnoside	2.324	0.139	9.081	**0.006**	3.035	***0.093***
Apigenin derivative 1	0.955	0.337	5.725	**0.024**	0.821	0.373
Methyl apigenin	1.222	0.311	7.642	**0.011**	1.950	0.177
Total apigenins	1.493	0.268	8.419	**0.008**	2.057	0.166
Total HPLC-phenolics	0.128	0.733	101.6	**<0.001**	1.460	0.240
Soluble CTs	0.260	0.660	19.30	**<0.001**	3.343	***0.081***
Cell wall bound CTs	0.514	0.479	2.179	0.151	0.147	0.704
Total CTs	0.039	0.850	12.62	**0.002**	2.553	0.124
Phenoloxidase acitivies						
PPO U g^{-1} FW	1.297	0.264	8.149	**0.008**	2.833	0.103
PPO U mg^{-1} protein	1.337	0.257	6.555	**0.016**	2.310	0.139
POD U g^{-1} FW	2.257	0.144	13.92	**0.001**	2.852	0.102
POD U mg^{-1} protein	2.291	0.141	11.00	**0.002**	2.552	0.121
Mechanical defenses						
Density of resin glands	0.183	0.670	39.11	**<0.001**	0.370	0.544
Hairiness of new stems	0.544	0.489	114.2	**<0.001**	0.284	0.204

Data were analyzed with Mixed model analysis (SPSS 17.0 for Windows). Statistically significant cases are in bold ($P \leq 0.0.05$) and close to significant cases are in italics ($P \leq 0.1$). $N=8$ for chemical defenses; $N=32$ for mechanical defenses

since fecundity correlates directly with the body weight of females (Haukioja and Neuvonen, 1985). We found no earlier studies conducted with woody plants wherein the effects of B on their resistance to herbivores were investigated. However, in a study conducted with annual soybean plants, B fertilization increased the resistance of the plants, such that the weights of soybean looper pupae, velvetbean larvae, and Mexican bean beetles were lowered

Table 3 The chemical and mechanical defenses of *Betula pendula* seedlings exposed to two treatments, boron and herbivory, and their four combinations

Defense	−B Control	−B Herbivory	+B Control	+B Herbivory
Phenolics (mg g^{-1} dw)				
DHPPG	**10.92±2.17*****	**26.04±2.17*****	**10.50±2.17*****	**19.68±2.17*****
(+)- Catechin	**0.685±0.07***	**0.899±0.07***	0.751±0.08	0.841±0.07
Cinnamic acid der 1	**0.096±0.01*****	**0.152±0.01*****	**0.096±0.01***	**0.125±0.01***
p-Cinnamic acid der 1	**0.205±0.03****	**0.295±0.03****	0.250±0.03	0.295±0.03
p-Cinnamic acid der 2	0.254±0.10	0.427±0.10	0.347±0.10	0.452±0.11
p-Cinnamic acid der 3	0.119±0.03	0.040±0.03	**0.048±0.03***	**0.135±0.03***
p-Cinnamic acid der 4	**0.195±0.03***	**0.303±0.03***	**0.163±0.03****	**0.327±0.03****
p-Cinnamic acid glycoside	1.085±0.18	1.539±0.18	**0.804±0.18****	**1.532±0.18****
Total cinnamic acids	**1.954±0.34***	**2.756±0.24***	**1.709±0.34****	**2.746±0.25****
Chlorogenic acid	1.207±0.16	1.612±0.16	**0.887±0.16*****	**1.791±0.16*****
Neochlorogenic acid	0.153±0.07	0.324±0.07	**0.097±0.07***	**0.364±0.07***
Coumaroyl astralgin	0.109±0.10	0.249±0.10	**0.039±0.10***	**0.298±0.10***
Myricetin 3-galactoside	3.708±0.59	2.822±0.59	**3.712±0.59***	**2.102±0.59***
Myricetin 3-glucoside+-glucuronide	**2.578±0.33****	**0.963±0.33****	1.939±0.33	1.429±0.33
Myricetin 3-arabinoside	0.401±0.06	0.379±0.06	**0.417±0.06***	**0.250±0.06***
Myricetin 3-rhamnoside	8.318±1.35	11.79±1.35	10.64±1.35	13.48±1.44
Total myricetins	15.01±1.75	15.96±1.75	16.70±1.75	19.17±1.75
Hyperin	**0.479±0.06***	**0.293±0.06***	0.368±0.06	0.214±0.06
Quercetin 3-glucoside+-glucuronide	0.723±0.15	0.793±0.14	0.710±0.15	0.680±0.14
Quercetin 3-arabinoside	0.403±0.05	0.345±0.05	**0.389±0.05***	**0.243±0.05***
Quercetin rhamnoside	**1.837±0.70*****	**4.749±0.70*****	**2.403±0.70****	**5.244±0.70****
Quercetin derivative	**0.438±0.17***	**1.016±0.17***	0.326±0.17	0.626±0.17
Total quercetins	**4.575±0.94****	**8.000±0.94****	**4.781±0.94***	**7.792±0.94***
Kaempferol 3-rhamnoside	0.596±0.04	**0.653±0.05**^{a}	**0.586±0.04****	**0.799±0.05**^{b**}
Apigenin derivative 1	**0.163±0.06***	**0.349±0.06***	0.159±0.06	0.243±0.06
Methyl apigenin	**0.962±0.31****	**1.952±0.31****	0.880±0.31	1.206±0.31
Total apigenins	**1.143±0.37****	**2.426±0.37****	1.044±0.37	1.478±0.37
Total HPLC-phenolics	**35.70±2.91*****	**57.84±2.91*****	**36.79±2.91*****	**54.27±2.91*****
Soluble CTs	73.18±6.57	59.84±6.57	**79.20±6.57*****	**46.83±6.57*****
Cell wall bound CTs	35.74±2.73	38.73±2.73	36.66±2.73	41.74±2.73
Total CTs	108.9±6.67	98.57±6.67	**115.9±6.67*****	**88.57±6.67*****
Phenoloxidase acitivies				
PPO U g^{-1} FW	1074±163	1275±173	**981.3±173****	**1757±173****
PPO U mg^{-1} protein	42.26±6.72	49.57±7.13	**39.70±7.13****	**68.38±7.13****
POD U g^{-1} FW	896.9±342	**1628±363**^{a}	**830.2±363****	**2770±363**^{b***}
POD U mg^{-1} protein	37.69±13.5	**61.91±14.3**^{a}	**36.51±14.3****	**105.7±14.3**^{b**}
Mechanical defenses				
Density of resin glands	**335.7±41.6*****	**604.4±41.5*****	**364.0±41.7*****	**605.3±42.4*****
Hairiness of new stems	**1.810±0.28*****	**3.919±0.28*****	**1.944±0.28*****	**4.276±0.28*****

Estimated mean±standard error (Mixed model analysis, SPSS 17.00). Statistically significant differences between B levels (LSD contrasts) are marked with different letters and significant differences between control and herbivory treatments with asterisk and bold face (*$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$). $N=8$ for chemical defenses; $N=32$ for mechanical defenses

when they fed on B fertilized plants (Beanland et al., 2003). The smallest insects also were produced on plants that received an optimal B:Fe:Zn -ratio for the growth of the plants (Beanland et al., 2003); this suggests that an optimal balance to the nutrition of plants may play a role in their defense against insect herbivores (see Phelan, 1996).

The induced chemical defensive responses of birch seedlings, which were measured from the new leaves that

Table 4 The effects of time, boron treatment and herbivory on the height growth of *Betula pendula* seedlings

Source of variance	Den Df	*F*	*P*
Time	90.0	524.9	**<0.001**
B treatment	6.14	0.083	0.783
Herbivory	83.5	81.93	**<0.001**
B treat x Herbivory	83.5	2.777	***0.099***
Time x B treat	90.0	4.668	**0.033**
Time x Herbivory	90.0	3.471	***0.066***
Time x B-treat x Herbivory	90.0	0.000	0.992
Initial height	87.7	243.0	**<0.001**

Data were analyzed with Repeated Mixed model analysis (SPSS 17.0 for Windows). Statistically significant cases are in bold ($P \leq 0.0.05$) and close to significant cases in italics ($P \leq 0.100$), $N=32$

developed after herbivory, also were affected by B fertilization; specifically both polyphenoloxidases (PPOs) and peroxidases (PODs) were induced, but only in the +B seedlings. Similarly the levels of two *o*-diphenolic compounds, putative substrates for PPOs and PODs, namely chlorogenic acid and neochlorogenic acids, were elevated in the +B seedlings. The resistance of the +B seedlings to subsequent herbivores also was probably enhanced by the priming of trees (see Ruuhola et al., 2007), since in our earlier studies of the mountain birch—autumnal moth system, we demonstrated that herbivory enhanced the oxidation of phenols by inducing PPO and POD activities (Ruuhola and Yang, 2006; Ruuhola et al., 2008; Anttila et al., 2010) that was related to a decrease in the growth rates of the most voracious fifth instars (Ruuhola et al., 2008). For other tree species, phenoloxidases also have been shown to play a role in their defense against herbivores (e.g., Tscharntke et al., 2001; Wang and Constabel, 2004); overexpression of PODs was even shown to enhance the resistance of primed hybrid poplars (Barbehenn et al., 2010).

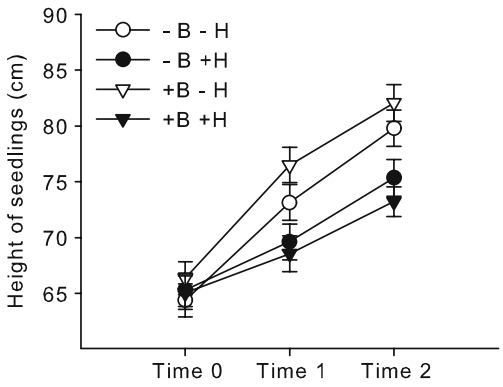


Fig. 4 The heights of *Betula pendula* seedlings at the beginning of the experiment (Time 0), after the pupation of *Epirrita autumnata* larvae (Time 1) and after a 2-week-recovery period (Time 2). The seedlings were exposed to two treatments, boron and herbivory, with their four combinations (−H=unwounded control; +H=herbivore-treated, –B=B unfertilized; +B=B fertilized seedlings). Data show ESmeans+S.E (Mixed model analyses, SPSS 17.00). $N=32$ per treatment

The B fertilized seedlings suffered from heavier defoliation than the –B seedlings, apparently due to compensation feeding of larvae, indicating that the B fertilization decreased the leaf quality for larvae. Quinones produced by phenolic oxidation are highly reactive compounds that readily bind many nutritionally important substances, especially essential amino acids, decreasing the plants' quality for their consumers (Felton et al., 1989). Many herbivores may increase their consumption when the nutritive value of their diet declines (Haukioja, 2003). Compensation feeding may, however, lead to the ingestion of toxic amounts of harmful secondary chemicals (Slansky and Wheeler, 1992; Haukioja, 2003); this can be seen as lowered utilization efficiencies, reduced feeding, a slowed growth rate, and increased mortality (Slansky and Wheeler, 1992). The effect of the greater defoliation of the +B seedlings was seen as a reduced height growth for these trees compared to the –B trees. The biomasses of seedlings for both B treatments were markedly decreased by herbivory, so that the defoliation percentage correlated negatively with the stem biomass. However, given more time, the B fertilized seedlings may have had better compensation abilities than the B deprived seedlings, so that they might make up for this reduced growth later in the present growing season or in future seasons.

In our earlier study, the induced resistance of birch trees was shown to increase the immunity of moths; that may explain the rapid population build-up in the increasing phase of population cycle *via* decreased rate of parasitism (Kapari et al., 2006). In our current study, the immunity of animals that were grown on the +B seedlings tended to be lower than that of animals grown on the –B seedlings, but if we had measured the effects of induced resistance on the next generation of moths (as in Kapari et al., 2006), the results might have been different.

Boron fertilization markedly increased the B concentration in birch leaves; however, the B concentration in the +B seedlings was substantially decreased (ca. 30%) by herbivore wounding with no significant decrease detected in the –B seedlings. The –B seedlings may not have been totally B deprived since the original peat plug may have contained some. The concentration of B in the leaves of the –B seedlings was low (7–8 μg g^{-1} dw), but was probably not yet below the critical deficiency limit, which in conifers is 4–5 μg g^{-1} dw (Braekke, 1979), since we did not detect marked decrease in the biomasses of –B seedlings. Neither did we detect visible deficiency symptoms. The average B concentration in the leaves of the +B seedlings was lower (20 μg g^{-1} dw) than the average value for the leaves of mature *B. pendula* trees (32 μg g^{-1} dw) that were collected

Table 5 The effects of boron and herbivore treatments on the weights of stems and branches and number of new lateral shoots of *Betula pendula* seedlings

	B treatment		Herbivory		B treatment x herbivory		Initial height	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Weight of current year stem and branches (g DW)	0.238	0.643	45.69	**<0.001**	0.070	0.792	5.436	**0.022**
Weight of previous year stem and branches (g DW)	2.021	0.159	58.36	**<0.001**	0.031	0.861	32.30	**<0.001**
Weight of whole stem and branches (g DW)	1.824	0.180	61.46	**<0.001**	0.013	0.908	29.20	**<0.001**
Number of new lateral shoots	0.181	0.686	8.939	**0.004**	0.390	0.534	12.87	**0.001**

Data was analyzed with the Mixed model analysis (SPSS 17.0 for Windows). Statistically significant cases are in bold ($P \leq 0.0.05$), $N=32$

from three different European countries (Hagen-Thorn and Stjernquist, 2005). However, our samples were collected early in the growth season, whereas those for the mature trees were collected in autumn.

Low B level in leaves of–B seedlings did not cause a phenolic accumulation nor the induction of PPOs or PODs in the leaves of birch seedlings. This is contrary to the results of earlier studies conducted with herbaceous species (Ruiz et al., 1998; Cakmak and Römheld, 1997; Camacho-Cristóbal et al., 2002). Similarly, in a study of the conifer, Norway spruce, B deficiency had a minor effect on phenolic contents; the level of condensed tannins (CTs) and monocoumaroyl-astragallin derivative were slightly induced in the B deficient trees (Rummukainen et al., 2007). Olive trees growing in the field with visible B deficiency symptoms also did not accumulate phenolics in their leaves, whereas potted B deficient olive seedlings cultivated in the growth chamber did (Liakopoulos and Karabourniotis, 2005). Perennial plants may store B in their stems and use these stores during bud burst (Lehto et al., 2004). Thus, they are not so vulnerable to a deficiency as herbal plants, especially under field conditions (Lehto et al., 2010). However, the silver birch has a limited ability to remobilize B from mature (Lehto et al., 2004) or senescing leaves (Ruuhola et al., 2011), suggesting that B is a rather immobile element in this species.

The levels of several phenolic compounds increased in the leaves of the herbivore-wounded seedlings, whereas the concentrations of other compounds decreased, reflecting changes in the allocation preferences towards defensively more effective compounds. For example, in the herbivore-treated seedlings, the level of 3,4′-dihydroxypropiophenone-3-β-D-glucopyranoside (DHPPG) increased, while the level of soluble condensed tannins (CTs) decreased. DHPPG is considered to be a defense against herbivory (Keinänen et al., 1999; Mutikainen et al., 2000; Huttunen et al., 2008), while CTs may decrease the defensive properties of other phenolics (Barbehenn et al., 2006). In earlier studies, the level of DHPPG also was increased by herbivory (Keinänen et al., 1999; Mutikainen et al., 2000; Huttunen et al., 2008), while the level of soluble CTs decreased in silver birch (Huttunen et al., 2008) and their precursors did in mountain birch (Anttila et al., 2010).

Herbivory caused the production of hairy new stems with a greater number of resin glands, whereas the B treatment had no effect on these mechanical defenses. Feeding by insect herbivores also has been shown to increase the density of trichomes in other studies (Mutikainen et al., 2000; Rautio et al., 2002; Dalin and Björkman, 2003; Valkama et al., 2005). Resin glands on birch stems have been identified as a mechanical defense against mammalian herbivores (Rousi et al., 1991; Tahvanainen et al., 1991; Laitinen et al., 2002). Based on the observations of this study, it seems that severe defoliation by insect herbivores induces the resistance of silver birch to both insect and mammalian herbivores.

In summary, B fertilized seedlings displayed a stronger induced resistance to herbivores and a stronger induction of

Table 6 The weight of stems and branches and number of new lateral shoots of *Betula pendula* seedlings exposed to two treatments, boron and herbivory, and their four combinations

	−B Control	−B Herbivory	+B Control	+B Herbivory
Weight of current year stem and branches (g DW)	**0.586±0.03*****	**0.388±0.03*****	**0.576±0.03*****	**0.363±0.03*****
Weight of previous year stem and branches (g DW)	**5.336±0.18*****	**3.951±0.18*****	**5.052±0.18*****	**3.730±0.18*****
Weight of whole stem and branches (g DW)	**5.921±0.20*****	**4.339±0.20*****	**5.629±0.20*****	**4.093±0.20*****
Number of new lateral shoots	**9.568±0.67***	**10.31±0.74***	11.82±0.73	11.78±0.74

Estimeated mean ± standard error (Mixed model analysis, SPSS 17.00). Statistically significant differences between control and herbivory treatments are marked with asterisk and bold face (LSD contrasts;$^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$), $N=32$

leaf phenoloxidases and their substrates than low-B counterparts; herbivores feeding on B fertilized seedlings showed reduced pupal weights and a slightly lower encapsulation response. These results indicate that adequate B nutrition of trees might play a role in the induced defense of birches against herbivory.

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Wheat Gene Expression is Differentially Affected by a Virulent Russian Wheat Aphid Biotype

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Abstract An improved understanding of the complex interactions between plants and aphids is emerging. Recognition of aphid feeding in plant tissues involves production of several defense response signaling pathways and downstream production of defense and detoxification compounds. Feeding by Russian wheat aphid, *Diuraphis noxia* (Kurdjumov), a serious pest of cereal crops worldwide, induces foliar deformity and chlorophyll loss during compatible wheat-*D. noxia* interactions. Experiments described here revealed significant differences in level and pattern of gene expression in defense response signaling and metabolic pathways between compatible and incompatible *D. noxia*-wheat interactions. The jasmonate (JA)-signaling genes *LOX, AOS,* and *AOC* were significantly more upregulated (~3- to 7 fold) in incompatible interactions than in compatible interactions (~2.5 to 3.5 fold) as early as 1 h post *D. noxia* infestation (hpi). Cellulose synthase, responsible for strengthening plant cell walls via cellulose production, was also more upregulated in incompatible interactions (4 to 7 fold) than in compatible interactions (1 to 3.5 fold). In contrast, glycolysis and citric acid cycle genes were significantly downregulated (~1.5 to 2 fold) in incompatible interactions and upregulated or less downregulated in compatible interactions from 6 to 72 hpi. Differences in expression of JA-signaling genes between feeding site tissues and non-feeding site tissues suggest that *D. noxia* defense response signals in wheat are restricted primarily to aphid feeding sites in the initial 6 hpi. This is the first report of differential upregulation of plant genes at 1 hpi in incompatible interactions involving aphid herbivory. Early wheat plant defense responses in incompatible *D. noxia* interactions at 1, 3, and 6 hpi appear to be important aspects of *D. noxia* resistance in wheat.

Key Words Aphids · *Diuraphis noxia* · Plant gene expression · qRT-PCR · *Triticum aestivum* · Wheat · Jasmonate signaling genes

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Introduction

Selection of a plant tissue substrate before and during the initiation of insect feeding depends on a suite of well-coordinated interactions between evolutionarily conserved protein(s) encoded by the attacking insect and the host plant that result in a compatible interaction, in which the insect successfully manipulates the host plant, or an incompatible interaction, in which the insect is unsuccessful (Kaloshian, 2004). Complex and dynamic interactions between plants and herbivorous insects have been documented for the response of plants to attack by herbivorous chewing insects (Walling, 2000; Kessler and Baldwin, 2002; Hatcher et al., 2004). However, much less is known about the more complex interaction between plants and insects with piercing-sucking mouthparts (Smith and Boyko, 2007).

Plant defense responses to herbivore-derived signaling compounds, including oral secretions, saliva, and oviposition fluids, represent herbivore-associated molecular patterns (HAMPs) that result in production of direct and

indirect chemical defenses (Mithofer and Boland, 2008). HAMPs that include fatty acid conjugates and peptide elicitors in oral secretions of Coleoptera, Lepidoptera, and Orthoptera have been documented (Doss et al., 2000; Schmelz et al., 2006; Alborn et al., 2007), but to date, elicitors secreted by aphids and other Hemiptera that contribute to these HAMPs remain unknown.

Aphids induce plant defense signaling pathways commonly activated by pathogens, and molecules eliciting these reactions may be directly synthesized by insects or may be products of endosymbiotic bacteria (Kaloshian and Walling, 2005). During these interactions, plants actively deploy defensive compounds to counter both aphid and pathogen attacks (Tjallingii and Hogen Esch, 1993; Walling, 2000; Kaloshian, 2004).

Recognition of aphid feeding in both dicot and monocot tissues involves generation of reactive oxygen species that elicit production of defense response signal cascades involving jasmonic acid (JA), salicylic acid (SA), ethylene (ET), abscisic acid (ABA), and giberellic acid; and downstream production of defense response and detoxification genes (see reviews by Couldridge et al., 2007; Smith and Boyko, 2007; Gao et al., 2008; Li et al., 2008). JA, OPDA (12-oxo-phytodienoic acid), and JAZ (Jasmonoyl-amino acid conjugates, governed by jasmonate ZIM [Zinc finger protein expressed in inflorescence meristem] repressor proteins) (Chini et al., 2007; Thines et al., 2007), control the expression of insect-induced JA early response genes (see review by Howe and Jander, 2008). Although cross-talk between JA and SA pathways occurs in response to aphid herbivory and phytopathogen infection (Balbi and Devoto, 2008; Kazan and Manners, 2008), biotrophic pathogens induce SA-mediated induced defenses, and necrotrophic pathogens and herbivorous insects induce JA/ET-mediated defenses (Kessler and Baldwin, 2002). However, considerable variation occurs in the JA-SA ratios of dicot and monocot foliage after aphid herbivory (Ellis et al., 2002a; Moran et al., 2002; Martinez De Ilarduya et al., 2004; Zhu-Salzman et al., 2004; Voelckel et al., 2004; Boyko et al., 2006).

In contrast to defense signaling genes, less information exists regarding expression of plant metabolic pathway genes elicited in response to insect herbivory. In general, expression of metabolism genes occurs over a more prolonged time course (days) than in defense signaling genes (hours). Divol et al. (2005) demonstrated greater expression of celery foliar genes associated with carbon and nitrogen metabolism in response to herbivory by green peach aphid, *Myzus persicae* Sulzer, at 7 days post infestation (dpi) than at 3 dpi. In incompatible interactions between wheat plants and the Russian wheat aphid, *Diuraphis noxia* (Kurdjumov), Botha et al. (2006) determined that genes involved in cell structure, cell maintenance, and photosynthesis that were down-regulated during initial infestation at 2 dpi were later up regulated at 8 dpi. Similar patterns of results have been observed in incompatible interactions between rice plants and the brown planthopper, *Nilaparvata lugens* Stahl, (Cho et al., 2005) and between wheat plants and the Hessian fly, *Mayetiola destructor* (Harris) (Zhu et al., 2008).

Diuraphis noxia is a serious pest of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) in all cereal-producing areas except Australia (Quisenberry and Peairs, 1998). During compatible interactions with *D. noxia*, susceptible wheat plants react to saliva injection by failing to unfurl leaves longitudinally around the main leaf vein, thus forming a tubular refuge that protects *D. noxia* from predators; plants suffer extensive leaf chlorophyll and carotenoid loss (Burd and Elliot, 1996; Heng-Moss et al., 2003). During incompatible interactions involving resistant plants, chlorophyll loss is minor, and *D. noxia* population growth is significantly reduced (Khan et al., 2009; Lazzari et al., 2009). Although several *D. noxia* resistance genes exist in barley and wheat, virulent *D. noxia* populations occur on all continents except Australia (Liu et al., 2010).

Results of recent studies involving the incompatible interaction of North American *D. noxia* biotype 1 and wheat plants containing the *Dnx* R gene demonstrated that an aphid HAMP exists in wheat, and consists of 16 carbon fatty acids; JA-, ABA-, and ET-signaling genes; and direct aphid defense genes (Smith et al., 2010). Upregulation of this HAMP increased significantly from 6 to 24 h post infestation (hpi), but expression of HAMP genes in compatible interactions (when present) was delayed until 48 to 96 hpi. In contrast, few carbon/nitrogen metabolism genes were expressed in incompatible interactions. On the basis of these results and those of Boyko et al. (2006), we hypothesized that incompatible *D. noxia* -wheat plant interactions are more dependent on unique JA-signaling transcripts than on SA-signaling transcripts for production of downstream *D. noxia* defense proteins, and that unique resistance gene transcripts are produced more rapidly in incompatible interactions than in compatible interactions.

The objectives of this study were to develop an improved understanding of the temporal nature of wheat defense signaling genes and metabolic pathway genes expressed during incompatible *D. noxia* -wheat plant interactions. A series of qRT-PCR experiments revealed unique significant differences in the expression patterns of several JA pathway genes and glycolysis and citric acid cycle pathway genes involved in plant carbon/nitrogen metabolism as early as 1 h after the onset of *D. noxia* feeding.

Methods and Materials

Plants and Insects The wheat cultivar 'Yumar' was used. Yumar carries the *Dn4* gene for resistance to *D. noxia*

biotype 1, which results in an incompatible interaction, but is susceptible to biotype 2, which results in a compatible interaction. Seeds of Yumar are currently maintained by the Plant Resistance Laboratory, Department of Entomology, Kansas State University, Manhattan, KS, USA. Biotype 1 aphids used originated from a culture established with aphids collected from Hays, KS, in 2004 and were provided courtesy of Dr. J. P. Michaud, Department of Entomology, Kansas State University. Biotype 2 aphids used were from an aphid colony originally collected near Briggs, CO, in 2004 and were supplied courtesy of Dr. Frank Peairs, Department of Bioagricultural Sciences and Pest Management, Colorado State University. Each biotype was cultured on biotype 1- and 2-susceptible 'Jagger' wheat plants in separate greenhouse rooms at 24:20°C day/night and a photoperiod of 14:10 h (L:D). The genetic identity of each colony has been verified yearly since 2005 in diagnostic plant differential assays at Manhattan, KS.

Experimental Design and Sample Collection Experimental treatments were a compatible interaction (*Dn4* plants infested by *D. noxia* biotype 2), an incompatible interaction (*Dn4* plants infested by *D. noxia* biotype 1), and control uninfested *Dn4* plants. Each treatment consisted of 6 plants grown in one pot. All plants were grown in an incubator at 25:22°C day/night and a photoperiod of 14:10 h (L:D). At the two-leaf stage, a mixture of 180 *D. noxia* biotype 1 or biotype 2 adults and large nymphs (30 per plant) were transferred to a small piece of paper that was placed in the center of the pot against the bases of the plant stems. A separate set of pots contained uninfested control plants. All pots then were covered with tight-fitting mesh cages. At 15 min after transfer, aphids had moved onto plants and begun settling. This event was designated as the zero time point. Results of previous EPG experiments indicate that there are no significant differences between *D. noxia* resistant- and susceptible plants for the mean time until initiation of the first *D. noxia* probe or the mean interval from the first probe to the first sieve element phase feeding event (Lazzari et al., 2009).

RNA Isolation and Real-Time PCR Aphids were completely removed from plant leaves with a camel's hair brush and a 1 × 1 cm section of leaf tissue from aphid feeding sites of each infested plant was collected at 1, 3, 6, 24, and 72 hpi. Expression of selected pathway genes in leaf tissue sections at aphid feeding sites was compared with expression in similar-sized leaf tissue sections from non-feeding sites in the same plants at 1, 3, 6, and 24 hpi. Using a previously developed RT-PCR protocol for the *D. noxia*-wheat system (Smith et al., 2010), there were two biological replicates for each set of tissues. Each replicate consisted of tissues pooled from 3 plants and two technical replicates for each genotype–tissue–post-infestation time combination sampled. Total RNA then was extracted from plant tissue sections using TRI reagent (Molecular Research Center Inc, Cincinnati, OH, USA) following the manufacturer's protocol. RNA quality and concentration were determined with a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). After purification using TURBO™ DNA-free (Ambion, Austin, TX, USA), 5 μg of total RNA were reverse transcribed into cDNA with a SuperScript III First-Strand cDNA Synthesis System (Invitrogen, Carslbad, CA, USA) following the manufacturer's protocol. Diluted (20x) cDNA and iQ™ SYBR Green Supermix (BIO-RAD) were amplified by Real-Time PCR (RT-PCR). The reaction was prepared in a 25-μl final mixture containing 12.5 μl 2X Supermix (2X reaction buffer with dNTPs, iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green 1, fluorescein, stabilizers); 10.5 μl cDNA; and 10 μM forward and reverse primers and performed under the following protocol: 5 min denaturation at 95°C, 40 cycles of 20 sec at 95°C, 20 sec at 58°C, and 30 sec at 72°C. Comparisons of product melting curves were used to confirm that each reaction generated a single PCR product.

On the basis of wheat expressed sequence tag sequences from GenBank, RT-PCR primers for SA and JA pathway genes, metabolism genes, and a cellulose synthase gene (Zhu et al., 2008) were designed using Beacon Designer software (Table 1). Wheat actin (AB181991) was used as an internal control. Genes tested for the involvement of SA in resistance included the shikimate pathway genes chorismate mutase (*CM*) and isochorismate synthase (*ICS*), involved in SA synthesis (Wildermuth et al., 2001); the phenylpropanoid pathway gene phenylalanine ammonia lyase (*PAL*), also involved in SA synthesis; and the SA marker pathogenesis-related 1 protein (*PR1*). Jasmonate pathway genes tested were Ω-3 fatty acid desaturase (*FAD*), which produces an 18:3 fatty acid precursor, lipoxygenase (*LOX*), allene oxide cyclase (*AOC*), allene oxide synthase (*AOS*), and 12-oxophytodienoate reductase (*12-OPR*).

Cellulose, a strengthening component of plant cell walls, is produced by a family of cellulose synthases (CES) (Scheible et al., 2001). Two members of the family (*CslA*, *CSLD2*) are upregulated in incompatible wheat interactions with either *M. destructor* or *D. noxia* (Liu et al., 2007; Smith et al., 2010). Ellis et al. (2002b) proposed that cellulose synthesis inhibition activates JA-dependent stress responses. *CES* expression was evaluated to determine its role in plant defense responses to *D. noxia* feeding and the relationship, if any, between *CES* expression and JA pathway gene expression.

Sixteen genes from the glycolysis, tricarboxylic acid (*TCA*), pentose phosphate, and amino acid synthesis pathways were examined. Glycolysis pathway genes examined were fructose 1-,6-biphosphate aldolase (*ALD*), glyceraldehyde-3-phosphate

Table 1 Primer sequences used to amplify selected defense response signaling pathway genes in wheat in response to feeding by the Russian wheat aphid, *Diuraphis noxia*

Accession	Gene name (abbreviation)	Primer sequences
EV254155	Isochorismate synthase (ICS)	CCACCAAACCAACCAACAGG//AGATTAAGAAATGAGGACGACGAG
DFCI	Chorismate mutase (CM)	CCGCCTATCCAGTATCCAAC//TCCATCACTTCCTTCCTTCAC
BJ281221	Phenylalanine ammonia lyase (PAL)	GGCGAGGAGGTGGACAAG//ACCAAGGCATCTTCTCTTCATC
AF384143	Pathogenesis-related protein (PR-1)	AACCTCGGCGTCTTCATCAC//TCGGTCCCTCTGGCTAGTTAG
CA616263	Omega-3 fatty acid desaturase (FAD)	CCAGAGAAGTCGGCTCCTC//TACTCGTTTCTGACTCACTTTGG
BT0089921	Lipoxygenase (LOX)	TGTTGATAGACTGGTGCTGTG//TGAGGATTAACGCTTAGGATCG
AY196004	Allene oxide synthase (AOS)	GAGCCCACCCACACCAAG//CGAGGAGCGAGGAGAAGTG
BJ241555	Allene oxide cyclase (AOC)	ATTCATTCAACACTGGTACAAGG//ATCTATTATTGCTCCTGCTAGTAG
CA650490	12-oxophytodienoate reductase (OPR)	GGAAGGCAACAAAGTGGTG//CGAATCTCTTAGGCAAATCTGG
CA699183	Cellulose synthase (CES)	CATTATGACCTTTGTGTTCTACTG//GCTGGAATATATACGCAACCC
AB181991	Actin (HK)	GGAAAATCAGTCTCGGTTCAG//TCATACAGCAGGCAAGCAC

dehydrogenase-like gene (*G3PDH*), cytosolic phosphoglycerate kinase (*PGK*), phosphoglycerate mutase (*PGM*), enolase (ENOL), and cytosolic pyruvate kinase (*PK*). TCA cycle genes evaluated were cytosolic malate dehydrogenase (*MD*), NAD^+-dependent isocitrate dehydrogenase (*ICD*), succinate dehydrogenase (*SD*), and α-ketoglutarate dehydrogenase (*OD*). Plastid enzymes $NADP^+$-dependent malic enzyme (*ME*) and phosphoenolpyruvate carboxylase (*PEPC*), and glucose-6-phosphate dehydrogenase (*G6PD*), a component of the pentose phosphate pathway, also were examined. Amino acid synthesis pathway genes evaluated were glutamine synthetase (*Gln-S*), pyrroline-5-carboxylate synthetase (*P5CS*), and methionine synthase (*Met-S*). Accession numbers, names, and functions of genes and their primer sequences are listed in Table 2.

Relative gene expression levels were determined as described by Pfaffl (2001). For ease of comparison, fold change values were converted to log2 values by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Differences between control and experimental groups were measured as relative values, and relative values greater than 1.5 fold in difference were considered biologically significant. Data for each gene were subjected to ANOVA using the SAS GLM procedure (SAS 2001). Significant treatment means were separated using the LSD at $P=0.05$.

Results

Salicylate Pathway Expression of ICS, an enzyme of the isochorismate branch of the SA pathway, decreased steadily in both plant-biotype interactions from 1 to 72 hpi (Fig. 1) except at 1 hpi in the incompatible interaction. The decrease was more pronounced in the compatible interaction; the 1.1-fold upregulation at 1 hpi and (−) 0.1-fold downregulation at 3 hpi were significantly greater than the (−) 1.1-fold downregulation at 72 hpi. In the incompatible interaction, differences were significant only between 1 hpi [(−) 0.3 fold] and 6 hpi [(−) 1.3 fold]. With the exception of the 1 hpi time interval, there were no significant differences in ICS expression between compatible and incompatible interactions.

In contrast to ICS, the SA-signaling downstream protein PR1 displayed an increasing temporal expression pattern that shifted from downregulation at 1, 3, and 6 hpi to upregulation at 24 and 72 hpi (Fig. 1). There were no significant differences in expression between compatible and incompatible interactions at any time point, but PR1 was significantly ($P<0.05$) more upregulated at 72 hpi than at all other time points in the incompatible interaction and significantly more upregulated at 72 hpi than at 1 or 6 hpi in the compatible interactions (Fig. 1). There were no significant differences in expression of CM, a second isochorismate SA pathway enzyme, in either plant-biotype interaction at any time point. All changes in expression levels were different by less than 1.5 fold (data not shown).

Expression of PAL, an enzyme in the phenylpropanoid branch of the SA pathway, was upregulated in both compatible (2.7 to 5.2 fold) and incompatible (4.4 to 5.8 fold) interactions at aphid feeding sites (Fig. 2). However, there were no significant differences between any time point or interaction comparisons.

Jasmonate Pathway Expression of FAD was upregulated in both incompatible (3.3 fold to 4.4 fold) and compatible (2.0 to 3.4 fold) interactions in tissues sampled from aphid feeding sites. However, differences in expression at each post-infestation time point were not significant (Fig. 2). Expression of LOX was not different over time within either compatible or incompatible interactions, but LOX upregulation was significantly ($P<0.05$) greater in incom-

Table 2 Primer sequences used to amplify selected metabolic pathway genes in wheat in response to feeding by the Russian wheat aphid, *Diuraphis noxia*

Accession	Gene pathway and name (abbreviation)	Primer sequences
Glycolysis		
AJ420778	Fructose 1-,6-biphosphate aldolase (ALD)	TGCCATCATCTGCCAGGAG//AAGGACGATCTCGGTGACG
AF273738	Glyceraldehyde-3-phosphate dehydrogenase-like gene (G3PDH)	CAGCCAACCTCCCATCTCC//TCCCGATCCTTCCGAAACC
X15232	Cytosolic phosphoglycerate kinase (PGK)	AGATTGTTCCTGCCACTGC//CTCAAACTCAAAGACTCCCATAG
EF210576	Phosphoglycerate mutase (PGM)	GCTGTAGCCAATTTTGTCG//ATCGGTCTGTTAAGCAAAC
AY335488	Enolase (ENOL)	TTAACTTCAAGGAGGAGAACAATG//TCAGCGGTCATCTTAGCATAG
AY623894	Cytosolic pyruvate kinase (PK)	GGCTCCCACGAGTACCAC//TCTAATCTCTGGACCCTTGG
Tricarboxylic acid		
AY236152	Cytosolic malate dehydrogenase (MD)	GCCGCATTTCCACTTCTC//ACCATAACCGCAACATTCAC
AY775790	NAD+dependent isocitrate dehydrogenase (ICD)	CTGAGAGGATTGCCAAATATGC//GACTCCAAGAACAAACCATCG
AF475119	Succinate dehydrogenase (SD)	CGAACCGCCCACTGTCTC//CTGGTAGCAAGAGGAATGGATATG
CK207322	NAD+−dependent malic enzyme (ME)	GTTCTCCCGATAATGCTTGAC//GCTAAGTATTCCTCTCCCTTCC
CD886657	α-Ketoglutarate dehydrogenase (2 - oxo-glutarate dehydrogenase, E1 subunit) (OD)	CTATGATCGTCGCCAGGTC//ATACCAGGAATCAGTGTCTCG
AJ007705	Phosphoenolpyruvate carboxylase (PEPC)	CGCTGCTCCCATTGATTCC//CCACTTAACAACACCAGATTACAC
Pentose phosphate		
AB029456	Glucose-6-phosphate dehydrogenase (G6PD)	CAGTGGTTCCTATGACAGTGG//GGTAGACAGATGGAGGCAATG
Amino acid synthesis		
DQ124211	Glutamine synthetase (Gln-S)	ATCTTCAAGGACCCGTTCAG//CAGCGTTGTATCTCTTGTTGG
AF022914	Pyrroline-5-carboxylate synthetase (P5CS)	CCGAAGGTAGATTCATTTCATC//AGTATCCGCTGACTTCTTATC
AF439723	Methionine synthase (Met-S)	GCTTGTCGTATCAACTTCCTG//TGACCAGCCAATGCCTTAG

patible interactions than in compatible interactions at both 3 and 6 hpi (Fig. 2).

AOS also was more upregulated in incompatible interactions (3.1 to 6.8 fold) than in compatible interactions (0.5 to 3.3 fold) across all post-infestation time points, and these differences were significant ($P<0.05$) at 6 and 24 hpi (Fig. 2). Within interactions, there were no differences in AOS expression in compatible interactions; however, in incompatible interactions, significantly more AOS was produced at 6 hpi than at 1 hpi. AOC demonstrated a similar pattern of expression. At 3, 6, and 72 hpi, upregulation in incompatible interactions (3.5, 3.7, and 0.9 fold, respectively) was significantly ($P<0.05$) greater than in compatible interactions (1.1, 1.7, and −1.1 fold,

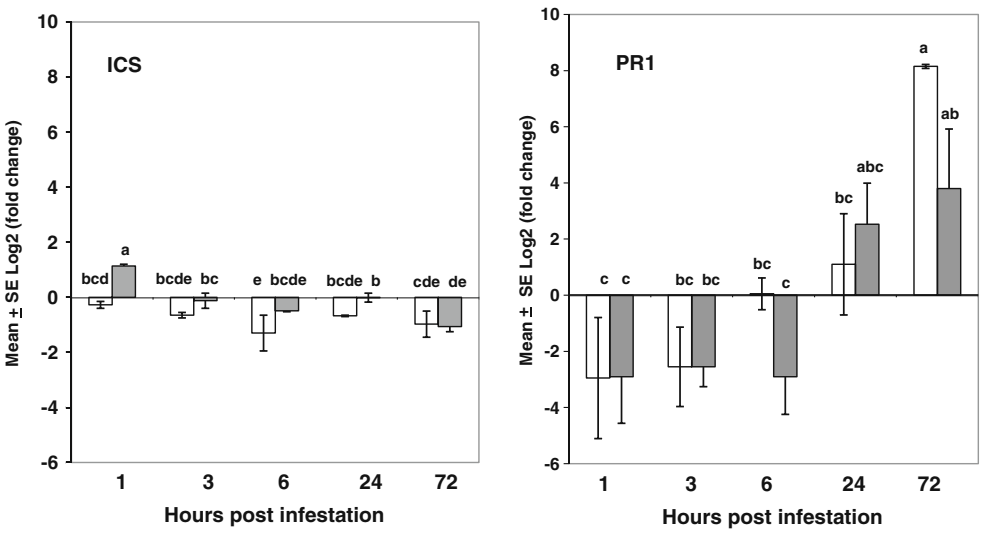


Fig. 1 Mean ± SE log2 (fold change) expression of wheat SA synthesis and SA-related genes induced by *Diuraphis noxia* feeding at 1, 3, 6, 24, and 72 h post infestation. White bars represent incompatible interactions; gray bars represent compatible interactions. ICS - Isochorismate synthase protein; PR1 - Pathogenesis-related protein. Means with a different letter differ significantly at $\alpha = 0.05$

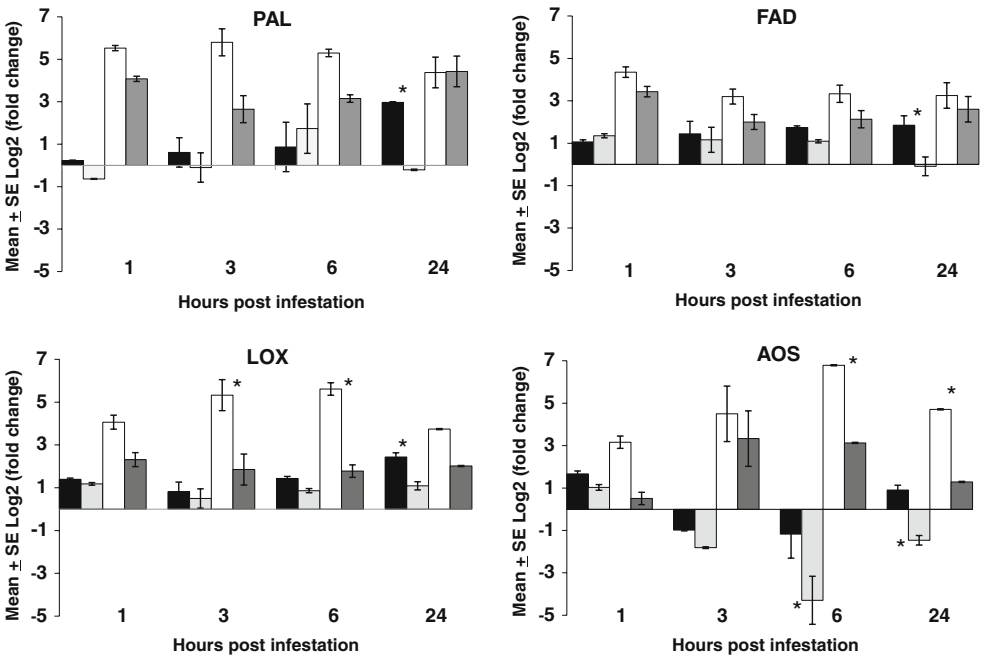


Fig. 2 Mean ± SE log2 (fold change) expression of wheat signaling genes at feeding sites and non-feeding sites of plant tissues from *Diuraphis noxia* compatible and incompatible interactions collected at 1, 3, 6, and 24 h post infestation. Black bars - non-feeding site tissues, incompatible interactions; light gray bars - non-feeding site tissues, compatible interactions; white bars - feeding site tissues, incompatible interactions; dark gray bars - feeding site tissues, compatible interactions. PAL - Phenylalanine ammonia lyase; FAD - Ω-3 fatty acid desaturase; LOX- Lipoxygenase; AOS - Allene oxide synthase. *=Treatment differences in mean expression within each feeding site or non-feeding site tissue comparison at each post infestation time differ significantly at $\alpha = 0.05$

respectively) (data not shown). Within incompatible interactions, expression was significantly greater at 3 and 6 hpi than at 24 or 72 hpi, and within compatible interactions, AOC expression was significantly greater at 1, 3, and 6 hpi than at 72 hpi (data not shown).

12-OPR was upregulated 1.9 to 3.9 fold in both incompatible and compatible interactions, but there were no significant differences in expression between time points or interactions (data not shown). Expression of CES was upregulated in both interactions at all post-infestation time points; within interactions there were no significant differences in expression at any time point. Upregulation was significantly ($P<0.05$) greater in the incompatible interaction (5.3 fold) than in the compatible interaction (1.3 fold) at 24 hpi (data not shown).

Gene Expression at Feeding Sites and Non-feeding Sites In general, expression of PAL, FAD, LOX, and AOS was much higher at *D. noxia* feeding sites than at non-feeding sites in both incompatible and compatible interactions (Fig. 2). As indicated previously, PAL expression at *D. noxia* feeding sites did not differ between incompatible and compatible interactions. Within each interaction, there were no significant differences in PAL expression in non-feeding site tissues between post-infestation times. However, PAL expression in non-feeding site tissues was significantly greater in incompatible interactions than in compatible interactions at 24 hpi (Fig. 2). FAD expression was similar to that of PAL, declining approximately 1 fold from 1 to 24 hpi (Fig. 2), and as indicated previously, expression in feeding site tissues did not differ. However, in non-feeding site tissues, FAD was significantly more upregulated in compatible interactions than in incompatible interactions at 24 hpi (Fig. 2).

The trend in LOX and AOS expression in feeding and non-feeding site tissues differed from that of PAL and FAD (Fig. 2). At *D. noxia* feeding sites, LOX upregulation peaked at significantly greater levels in incompatible interactions than in compatible interactions at 3 and 6 hpi (Fig. 2). In non-feeding site tissues, the trend in LOX expression was similar to that of PAL and FAD, with significantly greater expression between interactions occurring at 24 hpi. Further, at 24 hpi, there were no significant differences between LOX upregulation in non-feeding sites tissues of incompatible interactions and feeding sites tissues of compatible interactions (Fig. 2).

AOS expression in feeding site and non-feeding site tissues was similar to that of LOX (Fig. 2), with significantly greater upregulation in feeding site tissues in incompatible interactions than in compatible interactions at 6 and 24 hpi. In non-feeding sites tissues, AOS was significantly downregulated at 3 and 6 hpi within incompatible interactions compared with 1 and 24 hpi. Within compatible interactions, downregulation was significant only at 6 hpi. As with PAL, FAD, and LOX, AOS expression in non-feeding site tissues was significantly greater in incompatible interactions than in compatible interactions at 24 hpi. The significant increase of AOS in non-feeding sites tissues in incompatible interactions at 6 hpi was unique among the four signaling genes assayed.

Glycolysis Pathway In contrast to SA- and JA-signaling pathway genes, expression of all glycolysis pathway genes in feeding site tissues was greater in compatible interactions

than in incompatible interactions. Expression of PGM and PK was significantly more upregulated or less downregulated in compatible interactions than in incompatible interactions. PGM expression was greater at 6 and 72 hpi, whereas PK expression was greater at 24 and 72 hpi (Fig. 3). There were also within-interaction differences in expression of both genes. Although PGM expression in incompatible interactions was similarly downregulated at all time points, expression in compatible interactions was significantly upregulated at 6, 24, and 72 hpi compared with 1 hpi (Fig. 3). PK expression within incompatible interactions was also downregulated at all time points, and downregulation was significantly greater at 72 hpi than at 1 or 3 hpi (Fig. 3). In contrast, there were no differences in PK expression over all time points within compatible interactions (Fig. 3). In non-feeding site tissues, PGM and PK expression did not differ between interactions (data not shown).

Tricarboxylic Acid Cycle Pathway The trend in gene expression in the TCA cycle in feeding site tissues was downregulation during the early and late stages of *D. noxia* feeding and upregulation or decreased downregulation at 3 and/or 6 hpi. Downregulation was greatest for all genes at 72 hpi. There were no differences in expression either within or between interactions for ICD, MD, OD, and PEPC (data not shown). ME expression was significantly greater in incompatible interactions at 3 hpi than at 1, 24, or 72 hpi (Fig. 3). There were no differences in ME expression between the two interactions at all time points except 72

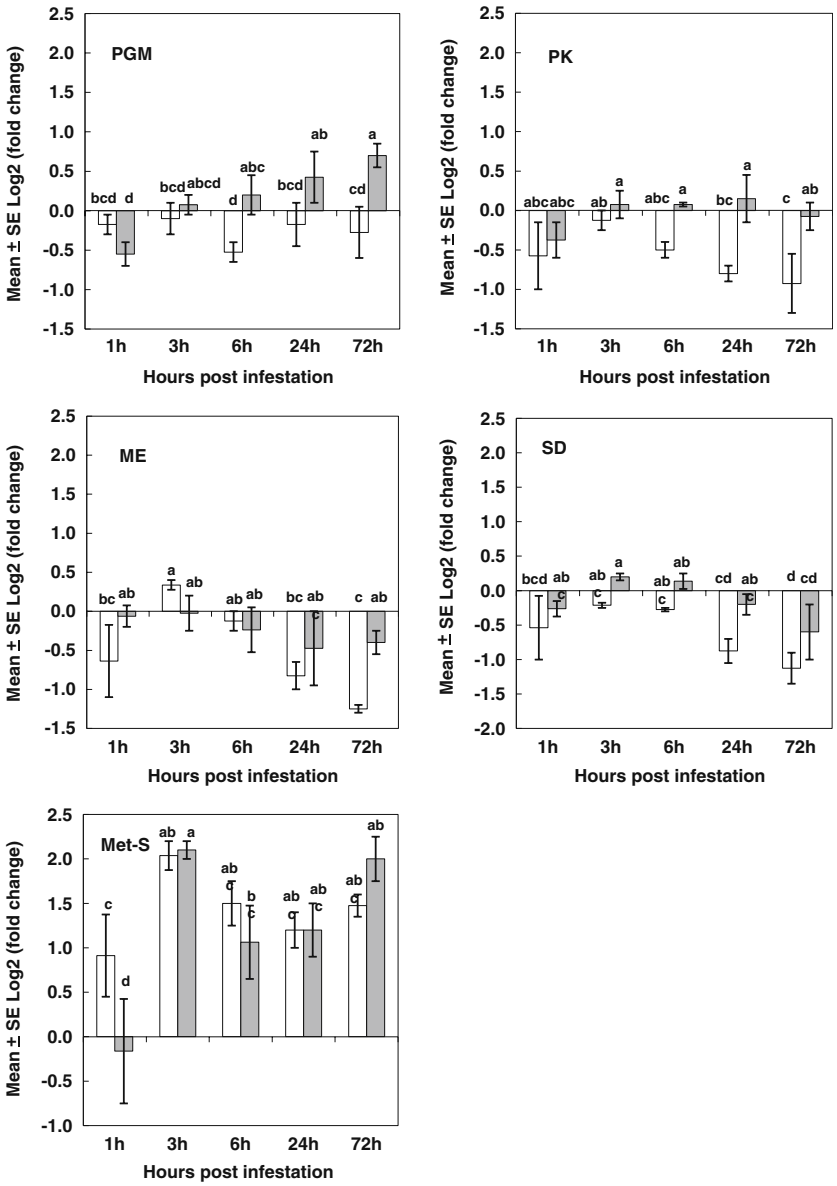


Fig. 3 Mean ± SE log2 (fold change) expression of glycolysis, tricarboxcylic acid, and amino acid synthesis genes induced by *Diuraphis noxia* feeding at 1, 3, 6, 24, and 72 h post infestation. White bars represent incompatible interactions; gray bars represent compatible interactions. Glycolysis - PGM (phosphoglycerate mutase), PK (cytosolic pyruvate kinase); Tricarboxcylic acid - ME (NAD^+ dependent malic enzyme), SD (succinate dehydrogenase); Amino acid - Met-S (methionine synthase). Means with a different letter differ significantly at $\alpha = 0.05$

hpi, when downregulation in incompatible interactions was greater than that in compatible interactions. SD expression did not differ between interactions at any time point, but within both interactions, expression was significantly greater at 3 and 6 hpi than at 72 hpi (Fig. 3). SD expression in tissues from non-feeding sites followed a similar trend in both interactions (data not shown).

Pentose Phosphate Pathway The pentose phosphate pathway gene G6PD was significantly more upregulated at 3 hpi (1.14 fold) than at 72 hpi ([−] 0.15 fold) in feeding site tissues in incompatible interactions (data not shown). There were no differences between any other time points or between either interaction at any post-infestation time points in non-feeding site tissues.

Methionine Synthase Synthesis Met-S upregulation increased significantly from 1 hpi to 3, 6, 24, and 72 hpi in compatible interactions and from 1 to 3 hpi in incompatible interactions (Fig. 3). At 1 hpi, Met-S expression was significantly greater in the incompatible interaction than in the compatible interaction. There were no differences in Met-S expression among treatments in tissues collected from non-feeding sites. Differences in expression levels of Gln-S and P5CS in tissues from both feeding and non-feeding sites were nonsignificant, both within and between interactions (data not shown).

Discussion

ICS and PAL participate in production of SA through conversion of chorismate in different branches of the salicylate pathway (Lee et al., 1995; Wildermuth et al., 2001). One branch involves ICS-catalyzed conversion of chorismate to isochorismate, and a second brings about conversion of phenylalanine to *trans*-cinnamic acid and benzoic acid via PAL. The lack of upregulation of ICS in either compatible or incompatible interactions at any time point suggests the isochorismate branch does not function in SA production in wheat in response to *D. noxia* feeding. Liu et al. (2007) reported similar results in wheat plants resistant to larvae of the Hessian fly, *Mayetiola destructor* (Harris). The early (1 to 6 hpi) expression of PAL at feeding sites involved in both compatible and incompatible interactions (Fig. 2) also suggests a lack of involvement of SA in wheat in resistance to *D. noxia* feeding. Finally, the delay in a significant increase in PR1 gene expression in incompatible interactions until 72 hpi suggests that PR1 is of limited importance in wheat defense responses to *D. noxia* feeding.

Although LOX, AOS, AOC, and CES were all significantly more upregulated in incompatible than in compatible interactions after *D. noxia* infestation, FAD expression, also often associated with JA synthesis, was no different between incompatible and compatible interactions. FAD and LOX are uniquely upregulated in incompatible interactions involving the wheat *Dnx* gene and *D. noxia* biotype 1 (Boyko et al., 2006; Smith et al., 2010). The absence of FAD expression may be related to the fact that in the present study, *D. noxia* interactions were compared using plants containing the *Dn4* resistance gene, not *Dnx*. Furthermore, Turner et al. (2002) determined that wound-induced JA production is regulated by the amount of substrate available to AOS. In incompatible interactions in our experiments, infested plants sustained minimal foliar damage, suggesting that reduced foliar chloroplast damage limited the release of fatty acids from chloroplast lipids for JA metabolism. Finally, plant mechanisms providing initial stimulation of the JA pathway remain largely unknown. However, factors such as SA-induced protein kinases and cell wall fragments now are known to activate production of various forms of methyl jasmonate (Howe and Jander, 2008; Bonaventure and Baldwin, 2010).

Microarray and related gene expression studies reveal differential expression of SA- and JA-signaling pathway genes in plants in response to biotic stress, as well as cross-talk between these and other defense response signaling pathways (Koornneef and Pieterse, 2008). However, our results fit insect herbivore-induced JA-signaling pathway models (Kessler and Baldwin, 2002; Howe and Jander, 2008) and demonstrate that several genes involved in methyl jasmonate synthesis play a major role in the very early events of wheat plant defense response signaling in response to *D. noxia* herbivory. In contrast, we found little evidence of the involvement of SA signaling in wheat plants in response to *D. noxia* feeding damage.

Diuraphis noxia feeding resulted in significant increases in expression of LOX (3 and 6 hpi) and AOS (6 and 24 hpi) in feeding site tissues collected from incompatible interactions compared to feeding site tissues from compatible interactions (Fig. 2). Similar response trends were not apparent in PAL or FAD expression. However, in non-feeding site tissues, PAL, FAD, and LOX expression in incompatible interactions did not increase significantly until 24 hpi. Increased AOS expression in non-feeding site tissues also occurred earlier, at 6 hpi. The lack of differences in PAL, FAD, and LOX expression in feeding site tissues at 24 hpi coupled with the significantly increased expression of these same genes and AOS in non-feeding site tissues at 24 hpi suggests that a systemic wheat leaf defense response signal may be transmitted from tissues damaged by *D. noxia* feeding to undamaged tissues within 24 hpi.

Li et al. (2002) demonstrated the existence of a mobile wound signal in tomato requiring JA perception for its

recognition in unwounded tissues, and Zhu et al. (2008) determined that wheat gene response to *M. destructor* larval herbivory is localized in both compatible and incompatible interactions. Future experiments measuring JA pathway gene expression in both feeding site and non-feeding site tissues at 24 hpi and beyond will be required to determine if such a mobile wound signal exists in wheat plants responding to *D. noxia* feeding.

Aphid feeding is known to enhance the plant nutrient "source-sink" by altering amino acid composition and level in host plant tissues (Telang et al., 1999; Forslund et al., 2000). However, in incompatible interactions, removal of amino acids and carbohydrates by *D. noxia* during source-sink rearrangements of feeding site tissues appears to be negated by the reduced plant upregulation of glycolysis and TCA cycle genes (Fig. 3) and upregulation of signaling genes in non-feeding site tissues on the same leaves (Fig. 2).

The level and pattern of expression of signaling genes differed from those of metabolic pathway genes in wheat plants responding to *D. noxia* attack. Upregulation of the jasmonate intermediates LOX, AOS, and AOC ranged from approximately 3 to 7 fold in incompatible interactions but upregulation was lower (approximately 2.5 to 3.5 fold) in compatible interactions. Similarly, CES was upregulated more (4 to 7 fold) in incompatible interactions than in compatible interactions (1 to 3.5 fold).

The trend and range of expression of glycolysis and TCA cycle genes were different from JA-signaling pathway genes. Expression of glycolysis and TCA genes (1.5 to 2 fold) was comparatively much less than JA pathway genes. In addition, PGM, PK, and ME were downregulated in incompatible interactions and upregulated in compatible interactions - a trend opposite to that observed in expression of JA pathway genes (Fig. 3). The upregulation of the PGM and PK glycolysis genes in compatible interactions increased degradation of sugars and other carbohydrates, likely resulting in stunted growth and development of susceptible plants. Zhu et al. (2008) noted similar results in incompatible interactions between wheat plants and *M. destructor* larvae.

One of the objectives of our experiments was to contrast patterns of expression of genes in signal pathways to those in metabolic pathways, in order to draw inferences about relationships between these pathways. Our results and those of others (Botha et al., 2006; Zhu et al., 2008) indicate that in gene expression studies conducted to date involving incompatible plant-insect interactions, plant carbon and nitrogen metabolism genes are initially downregulated and eventually upregulated days after infestation. In contrast, defense signaling genes are often highly upregulated in the initial hours after infestation, with expression declining continually over the course of 1 to 2 days after initial insect herbivory. Thus, this relationship between signaling and metabolic genes in wheat plants resistant to *D. noxia* appears to allow plant metabolism to slow down for several hours in order to expend the energy required to produce chemical and physical defenses resulting from defense response signal cascades.

Very early events occurring during interactions between aphids and host plants are poorly understood, especially incompatible interactions involving biotype or race-specific resistance (Thompson and Goggin, 2006). However, results of Lazzari et al. (2009) indicated that sustained ingestion of wheat phloem by *D. noxia* occurs within 1 h after initiation of feeding probes during compatible interactions and within 2 h after the beginning of feeding in incompatible interactions. Couldridge et al. (2007) demonstrated the expression of *Arabidopsis* esterase and extracellular glycoproteins in compatible interactions with the green peach aphid, *Myzus persicae* (Sulzer) at 2 hpi. Nevertheless, plant gene expression in response to aphid feeding during incompatible interactions has not been demonstrated to occur prior to 6 hpi (Li et al., 2008; as reviewed by Smith and Boyko, 2007). Thus, our results are of major significance because they are the first report of differential upregulation of plant signaling (LOX, AOC) and metabolism (Met-S) genes at 1 or 3 hpi in incompatible interactions involving aphid herbivory. Early defense responses of wheat plants in incompatible *D. noxia* interactions appear to be a very important aspect of *D. noxia* resistance in wheat.

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Attraction and Electroantennogram Responses of Male Mediterranean Fruit Fly to Volatile Chemicals from *Persea*, *Litchi* and *Ficus* Wood

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Abstract Trimedlure is the most effective male-targeted lure for the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). A similar response is elicited by plant substances that contain α-copaene, a naturally-occurring sesquiterpene. α-Copaene is a complex, highly-volatile, widely-distributed plant compound, and male *C. capitata* respond to material from both hosts (e.g., *Litchi chinensis*) and non-hosts (e.g., *Ficus benjamina*) that contain α-copaene. Avocado, *Persea americana*, recently was found to contain varying amounts of α-copaene in the bark and underlying cambial tissue. Short-range attraction bioassays and electroantennography (EAG) were used to quantify responses of sterile male *C. capitata* to samples of rasped wood from four avocado genotypes, *L. chinensis*, and *F. benjamina*. Gas chromatography-mass spectral (GC-MS) analysis was used to identify and quantify the major sesquiterpenes. Attraction and EAG amplitude were correlated, with *L. chinensis* eliciting the highest and *F. benjamina* the lowest responses. Responses to the avocado genotypes were intermediate, but varied among the four types. GC-MS identified 13 sesquiterpenes, including α-copaene, from all samples. Amounts of α-copaene in volatile collections from samples (3 g) ranged from 11.8 μg in *L. chinensis* to 0.09 μg in *F. benjamina*, which correlated with short-range attraction and EAG response. α-Copaene ranged from 8.0 to 0.8 μg in the avocado genotypes, but attraction and EAG responses were not correlated with the amount of α-copaene. Differences in enantiomeric structure of the α-copaene in the different genotypes and/or presence of additional sesquiterpenes may be responsible for the variation in male response. EAG responses were correlated with the amount of several other sesquiterpenes including α-humulene, and this compound elicited a strong antennal response when tested alone.

Key Words *Ceratitis capitata* · *Persea americana* · *Litchi chinensis* · *Ficus benjamina* · α-Copaene · Electroantennography · Bioassays · Diptera · Tephritidae

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Introduction

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), is considered the most polyphagous tephritid fruit fly species due to its broad range of host plants, primarily tropical and subtropical fruits and vegetables (Liquido et al., 1990). For this reason, *C. capitata* is presently ranked first among economically important fruit fly pests due to both damage to crops and costs of eradication (Hagen et al., 1981). This pest is established in Hawaii, and *C. capitata* outbreaks have occurred periodically in the continental US, including California and Florida. Rapid responses to these outbreaks led to declarations of eradication (Carey, 1996). Costs due to the 1980 California infestation included $59 million for chemical control, $38 million for quarantine and fumigation, $260 million in crop losses, plus an additional one-time cost of $497 million for construction of fumigation facilities (Burk and Calkins, 1983). It is estimated that it cost $26 million to eradicate the 1997 infestation in Tampa, FL (Stewart and Johanson, 1999). Highly effective detection and

monitoring systems along with weekly release of sterile males are important components of the preventative release program that has been established for *C. capitata* control and eradication (Dowell et al., 2000).

Jackson traps baited with trimedlure are the standard male-targeted traps for *C. capitata* (IAEA 2003). Trimedlure is a synthetic chemical that is highly attractive to males (Beroza et al., 1961). Trimedlure is not an insect-produced chemical nor has it been found in nature. Functionally, it acts as a pheromone that attracts sexually mature males, and has been coined a parapheromone (Cunningham, 1989). Natural substances have been found that attract male *C. capitata*, including angelica seed oil, which was used to bait traps during the *C. capitata* eradication in Florida in the 1950s (Steiner et al., 1957). The sesquiterpene α-copaene (8-isopropyl-1,3-dimethyl-tricyclo[4.4.0.0(2,7)]dec-3-ene) is the chemical found to be primarily responsible for this response, although other co-occurring chemicals may contribute to attraction (Jacobson et al., 1987). α-Copaene is a widely-distributed plant compound, and is found in many essential oils in addition to angelica seed oil (Flath et al., 1994a). While α-copaene is reported to be 2 to 5 times more attractive than trimedlure, difficulties in obtaining α-copaene in quantities sufficient for large scale trap deployment have prevented its use as field lures (Cunningham, 1989). Its concentration in natural substances generally is very low, and so there are no convenient plant extracts available as a practical source for this chemical (Flath et al., 1994b). The major problem with artificial synthesis of α-copaene lies in the chiral complexity of its 15-carbon structure, which makes synthesis not economically viable (Heathcock, 1966; Heathcock et al., 1967; Corey and Watt, 1973; Flath et al., 1994b). Essential oils containing α-copaene have been found to increase mating competitiveness of males that have been previously exposed to it, an approach termed aromatherapy since the males do not have to contact the oil to gain the benefit (Shelly, 2001a; Shelly et al., 2004). A recent study by Kouloussis et al. (2011) demonstrated that a mixture of volatile chemicals from citrus species (geraniol, α-pinene, limonene, β-myrcene, and linalool), which do not contain α-copaene, also increased the mating success of male medflies.

Male *C. capitata* responding to synthetic parapheromones or essential oils containing α-copaene tend to land near to but not make contact with the attractant (Steiner, 1957; Shelly et al., 2004). McInnis and Warthen (1988), however, observed that male *C. capitata* were attracted to and fed on the milky sap (latex) present in *Ficus benjamina* L., a non-host tree that they were using in research on mating behavior. In a survey of exudates from leaves and/or stems of 50 trees, they found levels of short-range attraction/feeding to exudates from *Litchi chinensis* Sonn., a *C. capitata* host, equal to responses to α-copaene purified from angelica seed oil and to trimedlure. Similar observations were made in field cage tests in which males aggregated and fed on the bark of some guava trees (*Psidium guajava*), but not all (active and inactive trees, respectively; Shelly and Villalobos, 2004). Chemical analysis found higher concentrations of α-copaene in active trees than in inactive trees. Males on active trees were observed to flatten their body against the bark and rapidly move their mouthparts up and down, frequently contacting the bark. Natural sources of α-copaene elicit a wide array of behaviors from male *C. capitata*. Studies of natural α-copaene sources may help determine the role of these phytochemicals in fruit fly biology as well as to quantify behavioral differences due to concentration of these chemicals (typically much lower in natural substrates vs. synthetic lures) and/or due to co-occurring chemicals (Raghu, 2004).

As part of a study on sesquiterpene content of tree bark and underlying cambial tissue, we found that avocado, *Persea americana* Mill., contained α-copaene and that levels were highly variable among different genotypes (R. R.H. unpublished data). Therefore, studies were conducted to determine if the α-copaene from *P. americana* is biologically active for male *C. capitata* and to evaluate the relationship between fruit fly response and sesquiterpene content. In addition to tests of avocado trees, studies were conducted with *L. chinensis* and *F. benjamina*, substrates that have been shown to be biologically active (McInnis and Warthen, 1988). We tested response of male *C. capitata* using short-range bioassays and electroantennography (EAG), and we quantified the sesquiterpene content. Information from this research will be used to further understand the role of phytochemicals in the biology of this fruit fly.

Methods and Materials

Insects Sterile male *C. capitata* were obtained from the Moscamed mass rearing facility (El Pino, Guatemala), where they were irradiated as pupae 2 d prior to emergence with 95 Gy of gamma radiation from a Co^{60} source. These are the temperature sensitive lethal strain flies (Franz, 2005) that are used for the preventative release program (Dowell et al., 2000), thus only males were obtained, and only virgin males were used for testing. Irradiated pupae were shipped initially to the USDA-APHIS Medfly Project (Sarasota, FL, USA), and then to the ARS Subtropical Horticulture Research Station (SHRS) in Miami, FL, USA. Holding conditions at SHRS consisted of a 12/12 h L/D photoperiod, 25±2°C, and 75±5% RH. After eclosion, adult flies were maintained in rearing cages (185×185×185 mm, ~100 flies per cage; BioQuip) and provided with water (agar blocks) and food (4:1 mixture of cane sugar and

yeast hydrolysate) *ad libitum* until collected for laboratory experiments. Flies used for all studies were 5 to 10 d-old and were sexually mature.

Sample Preparation Trees evaluated were *P. americana* (avocado, Lauraceae), *L. chinensis* (lychee, Sapindaceae), and *Ficus benjamina* (weeping fig, Moraceae) obtained from the USDA germplasm collection at the Subtropical Horticulture Research Station, Miami, FL. Samples were collected from six trees: 1-3) three half-sibling avocado trees (open pollinated seedlings), 14 yr-old (PAM 1-3), mother West Indian/Guatemalan hybrid cv. Melendez, father(s) unknown; 4) one Mexican race avocado cv. Seedless Mexican (PAMEX), 79 yr-old; 5) one *L. chinensis*, 78 yr-old; and 6) one *F. benjamina,* 77 yr-old. Samples were obtained by manually rasping the outer layers from freshly-cut tree branches (4.4±0.6 cm diam), which included bark and cambial tissue (defined as the lateral meristem including the vascular cambium and cork cambium in vascular plants, which carries the secondary metabolites). All samples were tested within 24 h of collection.

Binary Choice Tests Bioassays were performed to quantify the short-range attraction of sterile male *C. capitata* to samples using a modified version of the binary choice tests used by McInnis and Warthen (1988). All observations were carried out at room temperature as described above in rearing cages into which about 40 flies (average of 31 to 54 flies depending on the bait attractiveness) were introduced 1 h prior to the start of each experiment. Choice tests were initiated by introducing two Petri dishes (53 mm diam., 12 mm height) with substrates (sample bait or blank) positioned symmetrically (90 mm distance between the centers). After 15 min, the number of flies at each dish was recorded. Experiments were replicated four times, and the position of substrates reversed between replicates. Flies and Petri dishes were used only once, and cages were washed with acetone between experiments to eliminate potential residual chemicals. The attraction of each bait was tested initially against a blank (Petri dish with a cotton ball; 311 flies tested) before testing all possible two-at-a-time bait combinations (bait 1 vs. bait 2) (2336 flies tested).

Electroantennography EAG responses were recorded with a Syntech® system (Hilversum, The Netherlands) using methods described previously (Kendra et al., 2005). Test substrates were prepared by placing rasped samples (10 g) into hermetic bottles (250-ml) fitted with septum port lids. Samples were allowed to equilibrate overnight at 24°C to reach saturation. Freshly dissected antennal preparations were mounted between micropipette electrodes using conductive gel (Spectra 360; Parker Laboratories, Fairfield, NJ, USA) and placed under purified air flow. Using gas-tight syringes (VICI Precision Sampling, Baton Rouge, LA, USA), 2 ml samples of saturated vapor were withdrawn from the sample bottles, injected into the airstream, and presented to the antennae. There was a 2 min interval of clean air flow between sample injections to prevent antennal adaptation. EAG responses were measured initially in millivolts (peak height of depolarization) and then normalized to percentages relative to a standard reference sample, 100 µl 2-butanone saturated vapor (99% pure; Sigma-Aldrich, St. Louis, MO, USA) using EAG 2000 software. Normalization corrected for time-dependent variability in antennal performance, and all statistical analyses were conducted using normalized responses. For each sample, EAG responses were recorded from 5–10 insects, and response from each insect was the mean of three replicate measurements.

EAG response to α-humulene (>98% pure, Sigma-Aldrich, St. Louis, MO, USA) was tested as above. Test samples consisted of volumetric doses ranging from 125–4000 µl saturated vapor delivered as a series of two-fold increases in headspace volume. EAG responses were recorded from 10 insects, and response from each insect was the mean of three replicate measurements.

Gas chromatographic (GC)-EAD responses were recorded using Syntech® GC-EAD 2000 software (Hilversum, The Netherlands). One microliter samples of volatile collections from rasped samples from *L. chinensis* were injected into an HP6890 GC equipped with an Alltech Associates, Inc. column (Deerfield, IL, USA; 30 m EC-5, 320 µm diam, 25 µm film thickness) equipped with a 1:1 effluent splitter that allowed simultaneous FID and EAD detection of the separated volatile compounds. Helium was used as the gas carrier (1.2 ml. min^{-1}), and the injector was set at 250°C. Oven temperature was programmed from 50–130°C at 10°C. min^{-1} and from 130–220°C at 15°C. min^{-1}, then held at 220°C for 2 min. The column outlet for the EAD was held in a water-cooled humidified air stream (20°C) over a *C. capitata* antennal preparation.

Chemical Collection and Analysis Volatile chemicals were collected from rasped branch samples (6 g, 3 replicates per tree) using traps with super-Q as the adsorbent (Analytical Research Systems, Inc., Gainesville, FL, USA) as previously described (Heath and Manukian, 1992; Heath et al., 1993). Samples were spread in a cylindrical glass chamber (4.5 cm diam., 25 cm length), purified air was introduced into the chamber (1 L per min), and headspace volatiles collected for 15 min. Collector traps were cleaned by Soxhlet extraction using methylene chloride for 24 h and dried in a fume hood prior to use. Volatile chemicals were eluted from the super-Q adsorbent using 200 µl of high purity methylene chloride (99.5% pure; ACROS, Morris

Plains, NJ, USA). An aliquot of C_{16} standard (5 μg) was added to each sample for quantitative analysis. All chemical sampling was performed within 2 h of collecting the branch material.

Chemical extracts were analyzed using a ThermoQuest 2000 Trace GC (Austin, TX, USA). The column used was a fused silica, 25 m long, 0.25 mm I.D, DB-5MS phase (J&W Scientific, Agilent Technologies, Santa Clara, CA, USA), programmed from 50–130°C at 15.0°C min^{-1}, then from 130–220°C at 10.0°C min^{-1} and held at 220°C for 4 min. The column used in the GC interface to the mass spectrometer (Agilent Technologies 5975B, Santa Clara, CA, USA) was 25 m long, 0.25 mm I.D, DB-5MS phase (J&W Scientific, Agilent Technologies, Santa Clara, CA, USA), programmed from 40°C held for 2 min, 40–130°C at 10.0°C min^{-1}, then from 130–220°C at 20.0°C min^{-1} and held at 220°C for 4 min. Chemicals were identified tentatively by using the NIST mass spectral (MS) program version 2.0 d and NIST/EPA/NIH mass spectral library (NIST05) when reverse matches and matches were >950 and 900‰, respectively. The average reverse match for all identified chemicals was 980±14‰. The Kovats Retention Indexes (RI) were compared with previously published data or with the RI calculated from synthetic chemicals when commercially available [RI=1358, 1391, 1442, 1477 for α-cubebene (Bedoukian Research Inc., Danbury, CT, USA), α-copaene (Fluka analytical, Stenheim, Germany), β-caryophyllene (Sigma chemical Co, St. Louis, MO, USA), and α-humulene (Sigma chemical Co, St. Louis, MO, USA), respectively].

Statistical Analysis Bioassay results were analyzed using *Chi-square* tests (Statistica® 6.0 Software) to determine differences in attraction between choices. Differences in EAG responses and chemical content of the substrates were evaluated using non parametric Kruskall Wallis tests followed by Mann-Whitney tests. For each chemical, both the percentage of flies attracted and the EAG responses were compared to the quantity of chemical per substrate using Pearson correlation tests (Statistica® 6.0 Software). Regression analysis was used to describe the relationship between α-humulene dose and amplitude of EAG response (SigmaPlot® 10). Results are presented as mean ± SD unless otherwise noted.

Results

Binary Choice Tests Male *C. capitata* were more attracted to the rasped wood sample than to the paired blank regardless of the source. Percentages of tested males that were attracted by the baits were 48, 52, 35, 46, 38, and 49% for *L. chinensis*, PAM 1, PAM 2, PAMEX, PAM 3, and *F. benjamina* (χ^2=18.18, 30, 21, 34, 10, and 13 compared to blanks respectively, $P<0.01$). In binary choice experiments, flies made clear choices over each pair presented (Table 1), and overall pattern of preference from most to least attractive was *L. chinensis*, PAM 1, PAM 2, PAMEX, PAM 3, and *F. benjamina*.

Electroantennography Comparative EAG measurements (Fig. 1a) indicated that there were significant differences in amplitude of antennal response to fixed doses of vapor from the six rasped branch samples (z=2.193; $P<0.05$). EAG responses were correlated with the fly's preferences obtained in the bioassays (r^2=0.857, $P<0.05$), following the same hierarchy. Highest EAG response was recorded from *L. chinensis*, lowest response from *F. benjamina*, with

Table 1 Attraction (Mean ± SD) of sterile male *Ceratitis capitata* in binary choice tests between 3 g rasped branch samples (baits) from *Litchi chinensis* (litchi), *Persea americana* 'melendez' hybrids (Pam 1-3), *P. americana* "seedless Mexican" (PAMEX), and *Ficus benjamina* (ficus). Response recorded after 15 min

Bait 1 / Bait 2[a]	N[b]	% Responding[c]	% at Bait 1[d]	% at Bait 2[d]	χ^2	df	*P*
LITCHI / PAM 2	157	61.1	34.0±10.8	20.3±13.1	24.4	3	<0.001
LITCHI / PAMEX	216	58.8	57.2±15.2	3.2±3.2	1719.3	3	<0.001
PAM 1 / PAM 2	153	54.9	35.9±12.4	20.5±2.4	8.8	3	<0.05
LITCHI / PAM 3	131	53.4	43.6±17.5	6.4±4.1	581.4	3	<0.001
LITCHI / PAM 1	136	50.0	26.7±15.3	16.2±10.4	10.7	3	<0.05
PAM 2 / FICUS	141	49.6	50.2±16.7	3.4±2.5	55.5	3	<0.001
LITCHI / FICUS	194	47.4	47.3±6.2	0.4±0.7	89.0	3	<0.001
PAM 2 / PAMEX	161	46.6	36.4±4.9	14.1±13.0	36.7	3	<0.001
PAM 1 / PAMEX	140	40.7	34.6±17.1	13.3±10.7	18.2	3	<0.001
PAM 2 / PAM 3	139	38.8	38.3±11.1	2.7±3.2	88.5	3	<0.001
PAM 1 / FICUS	211	36.9	43.6±19.0	1.9±1.6	66.3	3	<0.001
PAM 1 / PAM 3	142	34.5	34.9±14.9	2.7±3.3	35.1	3	<0.001
PAMEX / PAM 3	135	34.1	25.0±14.4	12.1±9.8	8.6	3	<0.05
PAMEX / FICUS	126	27.0	23.0±12.2	5.7±5.3	22.3	3	<0.001
PAM 3 / FICUS	154	20.8	24.1±19.3	4.3±3.6	18.3	3	<0.001

[a] All choice tests replicated 4 times; position of baits reversed between replications

[b] Total number of flies used

[c] Total percentage of flies attracted to either bait after 15 min

[d] Percentage of responding flies attracted by each bait

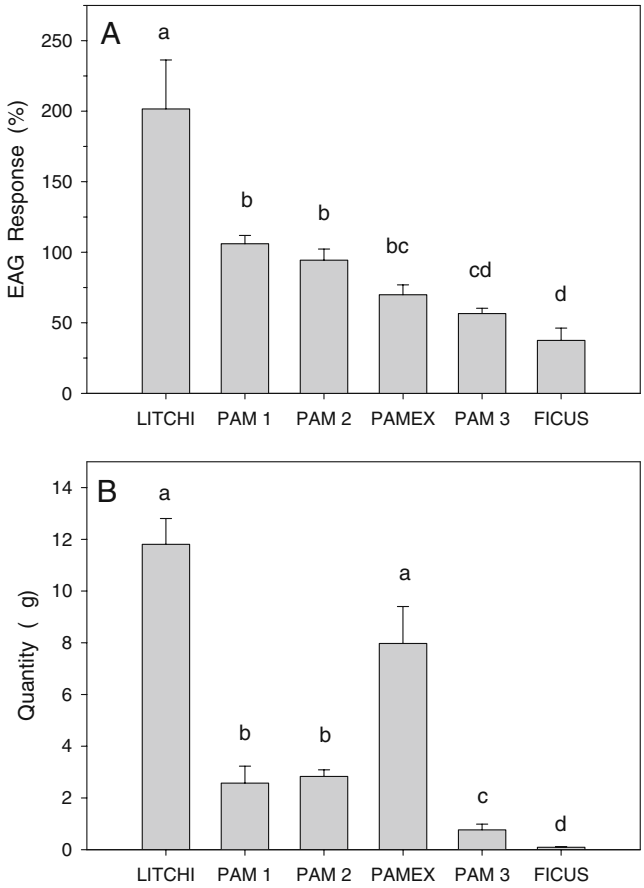


Fig. 1 **a**. Electroantennogram response (EAG) (mean ± SE) of sterile male *Ceratitis capitata* to fixed doses (2 ml saturated vapor) of volatiles emitted from 10 g rasped branch samples from *Litchi chinensis* (LITCHI), *Persea americana* 'Melendez' hybrids (PAM 1–3), *P. americana* 'Seedless Mexican' (PAMEX), and *Ficus benjamina* (FICUS). Responses were expressed as a percentage of the standard reference response (2-butanone). **b**. Quantity (mean ± SE) of α-copaene emitted from 6 g rasped branch samples, as determined by Super-Q collections and GC-MS analysis. Bars topped with the same letter were not significantly different (Mann-Whitney tests, $P<0.05$)

intermediate responses recorded from the various avocado samples. Among the avocado samples, EAG responses to PAM 1 and PAM 2 were significantly greater than response to PAM 3, with intermediate response to PAMEX.

Chemical Analysis A total of 51 volatile chemicals were isolated and identified by Super-Q collections and GC-MS analysis of the rasped wood samples. Of these, 13 compounds were found in common to all of the trees evaluated (Table 2). Chemical content (per gram) varied both qualitatively and quantitatively among the samples, with many of the chemicals detected only in trace amounts from *F. benjamina*.

EAG response was not correlated with the quantity of α-copaene emitted from the rasped wood samples (Table 2; $r^2=0.8036$, $P>0.05$; Fig. 1b) when results from all six trees were tested. Amount of α-copaene was highest in *L. chinensis* and lowest in *F. benjamina*, which was correlated with short-range attraction and EAG response. However, the lack of correlation was observed among the avocado samples, with low level response to PAMEX in spite of a high level of α-copaene. EAG response was correlated with the quantities of four other chemicals, all sesquiterpenes, specifically α-humulene (also known as α-caryophyllene) ($r^2=0.9341$, $P<0.01$), β-caryophyllene ($r^2=0.9261$, $P<0.01$), and both unidentified compounds (RI=1422, 1447) (suggested to be *cis*- and *trans*- enantiomers of α-bergamotene by the NIST library) ($r^2=0.8352$, $P<0.05$ and $r^2=0.691$, $P<0.05$, respectively). GC-EAD analyses were conducted to determine whether these chemicals elicited an antennal response. The analysis showed that α-copaene and α-humulene triggered the two main antennal responses.

α-Humulene was evaluated further by EAG. There was a relationship between dose of α-humulene and amplitude of EAG response, which was best fit by regression with a hyperbolic model (Fig. 2; $R^2=0.994$; $y=53.69x/0.30+x$; where x is the substrate dose in milliliters saturated vapor, and y is the normalized EAG response).

Discussion

Male *C. capitata* showed distinct preferences among the rasped branch samples in the laboratory bioassays. No calling behavior was observed in males responding to the samples, at least in the short time period of the bioassay, so males were responding to volatile chemicals from the rasped material. Attraction to these substrates was correlated directly with the amplitude of EAG responses. In combination, these results suggest that olfactory cues emitted from branches may play a role in location of trees where males are likely to encounter potential mates or in site selection for male aggregations. Males of *C. capitata* form mating aggregations, known as leks, which are by definition not resource-based. Leks are characterized by a loose aggregation of males performing sexual calling (pheromone emission) on the foliage of both host and non-host tree species (Steiner et al., 1957; Beroza and Green, 1963; Prokopy and Hendrichs, 1979; Whittier et al., 1992; Shelly and Whittier, 1996; Nishida et al., 2000; Shelly, 2004). Males hang on the undersurface of leaves, defending them as mating territories and displaying pheromone calling behaviors to attract potential mates (Arita and Kaneshiro, 1986). Olfaction has been shown to play a role in tree selection for lekking, but there are conflicting conclusions concerning male-produced aggregation cues (see Kaspi and Yuval, 1999; Shelly, 2001b). The nature of the olfactory cues used by male *C. capitata* for lek site selection remains elusive, and identification of

Table 2 Quantity (μg, mean±SD) of volatile chemicals emitted from 3 g rasped branch samples from *Litchi chinensis* (litchi), *Persea americana* 'melendez' hybrids (PAM 1-3), *P. americana* "seedless Mexican" (PAMEX), and *Ficus benjamina* (ficus). Volatiles were isolated by super-Q collection and GC-MS analysis. Trace = Quantity <0.05 μg

Chemical	RI[a]	LITCHI	PAM 1	PAM 2	PAMEX	PAM 3	FICUS
Unidentified	1049	0.02±0.01	0.43±0.17	0.20±0.04	0.33±0.09	0.14±0.05	0.07±0.04
δ-Elemene	1344[1]	0.07±0.04	1.91±0.73	0.48±0.05	0.11±0.02	1.17±0.55	Trace
α-Cubebene	1357[*]	0.21±0.01	3.04±1.06	2.83±0.70	0.06±0.12	1.98±0.91	0.01±0.01
α-Copaene	1391[*]	11.81±1.73[b]	2.58±1.13[b]	2.83±0.45[b]	7.97±2.48[b]	0.76±0.39	0.09±0.04
Unidentified	1422	0.66±0.74	0.24±0.12	0.14±0.05	0.06±0.05	0.16±0.06	0.02±0.01
β-Caryophyllene	1439[*]	7.89±2.33	3.18±0.91	0.65±0.55	2.58±0.41	1.61±0.60	0.05±0.06
Unidentified	1447	0.24±0.04	0.20±0.05	0.09±0.03	0.05±0.01	0.13±0.05	0.01±0.00
α-Humulene	1475[*]	1.79±0.27	0.31±0.10	0.14±0.05	0.19±0.04	0.27±0.12	0.01±0.02
Alloaromadendrene	1480[2]	0.12±0.05	1.72±0.47	0.87±0.05	0.39±0.10	1.20±0.65	Trace
Muurolene	1487[3]	0.28±0.09	0.48±0.23	0.26±0.09	0.41±0.12	0.09±0.04	0.01±0.01
Eremophilene	1510[3]	0.23±0.03	0.15±0.11	0.10±0.01	0.12±0.03	0.23±0.13	0.01±0.00
Cadinene	1529[1,3]	1.14±0.26	1.03±0.43	0.52±0.16	1.49±0.41	0.28±0.11	0.02±0.01
Unidentified	1547	0.07±0.03	0.10±0.04	0.08±0.04	0.06±0.03	0.05±0.03	0.01±0.01

[a] Kovats Retention Indexes obtained from analysis on a DB-5MS column

[b] Co-eluting with α-ylangene

[*] RI confirmed with synthetic chemicals

[1] Singh et al. 2007; [2] Tokushima et al. 2010; [3] Ibrahim et al. 2010

those volatile chemicals may provide a new approach for management of pest populations. Previous investigations have focused on α-copaene as a lekking cue (Nishida et al., 2000; Shelly et al., 2008). However, leks have been observed in both *L. chinensis* and *F. benjamina* (Whittier et al., 1992; Shelly and Whittier, 1996; Shelly, 2004), two trees that differ greatly in α-copaene content. Although not a preferred host, some avocado varieties are known to be susceptible to *C. capitata* (Back and Pemberton, 1918; Willard and Mason, 1929; Oi and Mau, 1989; De Graaf, 2009), but the amount of α-copaene in those varieties is unknown.

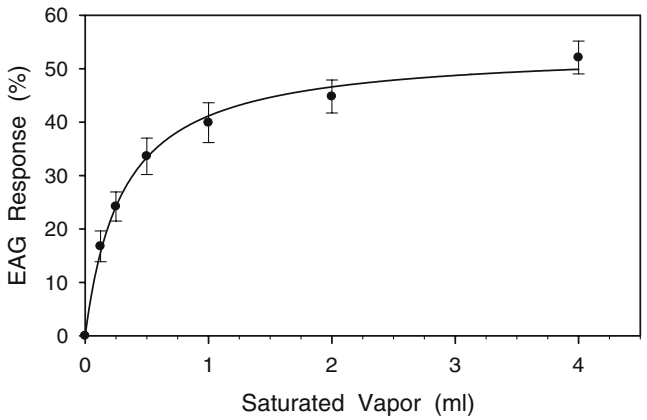


Fig. 2 Electroantennogram response (mean ± SE) and hyperbolic regression of sterile male *C. capitata* to a range of doses (saturated vapor) of α-humulene. Responses are expressed as a percentage of the standard reference response (2-butanone)

We identified 51 different volatile chemicals from these 6 trees. Among these secondary metabolites, 13 compounds were common in all the trees tested. These volatiles were mostly sesquiterpenes, which are widely spread in the plant kingdom and are often used by herbivorous or parasitoid insects to find their host trees. *Ceratitis capitata* is a generalist herbivorous pest attacking a wide range of hosts, more than 300 plant species (USDA, 1983). Thus, these flies may be attracted by one or a few volatile compounds better than by a mixture of several chemicals in a specific ratio. The amount of α-copaene in the rasped wood was not directly related with the fly preferences in our study. Indeed, flies showed less preference and lower antennal response for seedless Mexican (PAMEX) avocado, which released three times more α-copaene than another variety PAM 1. α-Copaene represented 31% of the sesquiterpenes in PAMEX but only 12% in PAM 1. There may be additional differences in isomeric and/or enantiomeric structures from the different trees that need to be evaluated further.

There were four sesquiterpenes for which the amount present was correlated with EAG response; specifically, α-humulene, β-caryophyllene, and two unidentified chemicals (RI=1422 and 1447). Beroza and Green (1963) reported β-caryophyllene as a *C. capitata* attractant, but Warthen and McInnis (1989) were unable to reproduce their results. It has been suggested that presence of α-copaene as a contaminant in their sample may explain the observed attraction (Cossé et al., 1995). Grové et al. (1998) subsequently showed that

male *C. capitata* were not attracted by β-caryophyllene in field tests. In our results, the amount of β-caryophyllene was much higher in PAMEX and PAM 3 than in PAM 2, the latter of which was one of the most attractive trees in this experiment. Thus, our study supports the hypothesis that β-caryophyllene is not a *C. capitata* attractant. The two other correlated chemicals were unidentified, but are suspected to be *cis*- and *trans*-α-bergamotene based upon NIST library matches. It was not possible to confirm the identification since α-bergamotene enantiomers were not available commercially, so they were not evaluated further; but additional studies were conducted with α-humulene.

α-Humulene is widely distributed in the plant kingdom (>40 families) and is known as an attractant for several insect families (Heteroptera: Pentatomidae and Pyrrhocoridae; Hymenoptera: Braconidae and Ichneumonidae; Coleoptera: Buprestidae, Cerambycidae, and Curculionidae; Lepidoptera: Torricidae; and Neuroptera: Chrysopidae) (Flint et al., 1979; Elzen et al., 1984; Ndiege et al., 1991; Farine et al., 1992; Hou et al., 1997; Weissbecker et al., 2000; Bedard et al., 2002; Carrasco et al., 2005; Crook et al., 2008; Yasui et al., 2008). This volatile chemical triggered a dose-dependent antennal response in sterile males, which reached a plateau at ~2 ml of saturated vapor. Hyperbolic equations have been shown to serve well for characterizing EAG dose-response relationships; the generic equation is expressed in the form $y=ax/b+x$, where the coefficients a and b indicate maximal EAG response (receptor saturation) and receptor binding affinity, respectively (Kendra et al., 2009). The normalized EAG response of *C. capitata* to α-humulene is much higher than the EAG response of *Anastrepha suspensa* (Loew) to putrescine (1,4-diaminobutane) (Kendra et al., 2008), a component of a synthetic attractant for this fruit fly. Although not attractive alone, putrescine has been shown to be a strong synergist to ammonium acetate for capture of both *A. suspensa* (Kendra et al., 2008) and *A. ludens* (Loew) (Heath et al., 1995, 2004). Consequently, α-humulene may play a role either directly as an attractive compound or indirectly as a synergist to α-copaene and/or the two unidentified chemicals (RI=1422 and 1447). Additional studies are needed to evaluate α-humulene as a potential semiochemical for male *C. capitata.*

Results of this study may lead to a better understanding of the role of phytochemicals in the biology of *C. capitata*. The biological significance of these compounds, which appear in both host and non-host plants, is still unknown. The phytochemical methyl eugenol is a parapheromone for the oriental fruit fly, *Bactrocera dorsalis* (Mendel). In addition to being a strong attractant, males that feed on methyl eugenol-containing substrates gain a significant mating advantage in both increased signaling and production of a more attractive pheromone blend (Shelly and Dewire, 1994) through sequestration of methyl eugenol metabolites (Nishida et al., 1988). Methyl eugenol is also a parapheromone and feeding attractant for the guava fruit fly, *Bactrocera correcta* (Bezzi). Tokushima et al. (2010) found that methyl eugenol metabolites, which were different from those sequestered by *B. dorsalis*, and also sesquiterpenes including α-humulene and β-caryophylene were sequestered in the male pheromonal rectal glands. In contrast, male *C. capitata* do not interact with essential oils that contain α-copaene, and unless the α-copaene is present in a low concentration, males tend to arrest near the essential oil but not contact it (e.g., Steiner et al., 1957). Males exposed to volatile chemicals from ginger root oil or orange oil slightly increase their pheromone signaling but do not produce a more attractive pheromone blend (Shelly, 2001a, Papadopoulos et al., 2006). Females, however, are more attracted to dead aromatherapy-treated males, extracts from hexane-rinsed aromatherapy-treated males, and treated males with very short (30 sec) exposure periods than similar presentations from non-treated males (Shelly, 2006, Shelly et al., 2007). Thus, it is hypothesized that the phytochemical odors interact with the male exoskeleton and provide chemical cues to females that are used for mate selection. Unlike essential oils, males have to make physical contact with fruit or bark substrates to gain a mating advantage (Shelly et al., 2007). Comparisons of chemical profiles of avocado, lychee, and ficus wood with profiles from essential oils, as well as biological effects of these substrates may provide new information on phytochemicals that are biologically active for *C. capitata*.

Although only sterile males were used in our studies, previous studies have shown similar response patterns to parapheromone-baited traps by wild and sterile males (e.g., Wong et al., 1982; Jang et al., 2010; N.D.E. unpublished data). Additionally, exposure to substances that contain α-copaene enhances mating success of both wild and sterile males (Shelly, 2001a). However, additional tests with wild males are needed to determine if they show similar behaviors in response to these substrates. Tests of short-range attraction or quantification of EAG response of sterile males to natural substrates may provide new methods to assess quality of mass-produced sterile males as the ability to locate plant-based bioactive chemicals may contribute to the effectiveness of released males in finding lek sites and competing with wild males.

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RAPID COMMUNICATION

Host Plant Invests in Growth Rather than Chemical Defense When Attacked by a Specialist Herbivore

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Abstract Plant defensive compounds may be a cost rather than a benefit when plants are attacked by specialist insects that may overcome chemical barriers by strategies such as sequestering plant compounds. Plants may respond to specialist herbivores by compensatory growth rather than chemical defense. To explore the use of defensive chemistry vs. compensatory growth we studied *Brugmansia suaveolens* (Solanaceae) and the specialist larvae of the ithomiine butterfly *Placidina euryanassa,* which sequester defensive tropane alkaloids (TAs) from this host plant. We investigated whether the concentration of TAs in *B. suaveolens* was changed by *P. euryanassa* damage, and whether plants invest in growth, when damaged by the specialist. Larvae feeding during 24 hr significantly decreased TAs in damaged plants, but they returned to control levels after 15 days without damage. Damaged and undamaged plants did not differ significantly in leaf area after 15 days, indicating compensatory growth. Our results suggest that *B. suaveolens* responds to herbivory by the specialist *P. euryanassa* by investing in growth rather than chemical defense.

Key Words *Brugmansia suaveolens* · Compensatory growth · Lethal plant defense paradox · *Placidina euryanassa* · Tropane alkaloids

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Introduction

The lethal plant defense paradox (Fordyce, 2001) postulates that although plant secondary metabolites provide a benefit against generalist herbivores, they may be a cost to plants under attack by specialists. Specialist feeders can tolerate or detoxify plant defensive compounds, sometimes sequestering them for defense or mating, or using them as cues for host finding (Trigo, 2011). Decreasing levels of chemical defenses in response to damage by specialists may be an adaptive plant response (Lankau, 2007). Alternatively, herbivory by a specialist might result in tolerance (Strauss and Agrawal, 1999) such as compensatory growth, which allows damaged plants to replace tissues lost to herbivores (Belsky, 1986).

We investigated the defense paradox using *Brugmansia suaveolens* (Solanaceae), which produces tropane alkaloids (TAs) and is attacked by larvae of the specialist ithomiine butterfly *Placidina euryanassa* (formerly *Placidula euryanassa*) (Freitas et al., 1996). Larvae of *P. euryanassa* show warning coloration, and sequester TAs that remain in the pupae and adults (Freitas et al., 1996), thus conferring protection against predators, such as birds and ants (Arab and Trigo unpublished results). The TA scopolamine is inducible in the leaves after artificial damage, and negatively affects larval performance of the generalist fall armyworm *Spodoptera frugiperda* (Alves et al., 2007).

We hypothesized that leaves of *B. suaveolens* would not induce TAs when attacked by *P. euryanassa*, and would invest in compensatory growth as an alternative defense strategy. To test our hypothesis we examined the levels of TAs in leaves 24 hr and 15 d after damage, and leaf area 15 d after damage. We used these time intervals based on the timing of TA induction and relaxation after artificial damage (Alves et al., 2007).

Methods and Materials

Organisms Several stems of *B. suaveolens* (*ca.* 15 cm high) were cut from individuals (hereafter genets) at the Serra do Japi forest, Jundiaí, SP, Brazil. Four stem clones of each genet were rooted in 5-L plastic pots filled with soil in a greenhouse at 25±3°C and 12:12 hr L:D photoperiod, and allowed to grow for 45 d. Afterwards, each stem was about 30 cm high and had 4 to 12 fully expanded leaves. The 4th instars of *P. euryanassa* were obtained from one egg batch that was collected from leaves of *B. suaveolens* at the same site.

Treatments The effect of 24 hr larval feeding on the level of TAs in *B. suaveolens* was assessed by using 13 genets. For the feeding treatment, 4th instars of *P. euryanassa* were confined individually within a clip-cage at the half distal portion of the leaves 1 and 4 (assigned from the apical meristem). The half distal portion was considered half of the leaf length without petiole. Clip-cages were made from the tops of Petri dishes (5 cm diam) attached to either side with a rubber band. Each cage was supported by a wooden stake to avoid weighing down the leaves. After 24 hr, clip-cages and larvae were removed. At this time, the half distal portion of each leaf was consumed. The first four expanded leaves with petioles (the two damaged ones, i.e., leaves 1 and 4, and the two undamaged ones, i.e., leaves 2 and 3) were harvested. The damaged and undamaged leaves were taken together for TA analyses (see below) since they showed no significant differences in TA concentration (data not shown). For the undamaged control treatment, plants had the same clip-cages, without larvae in the cages, and leaves 1 to 4 were harvested. The harvested leaves were oven dried at 50°C for 2 d, ground, and maintained at −10°C until TA extraction and analysis.

The concentration (μg/g) data for each TA were ln (x+1) transformed to meet the assumption of homoscedasticity and compared between the treatments using a one-way randomized block MANOVA, since the four TAs are not independent variables; they share the same biosynthetic pathway (Hashimoto et al., 1991). The genets were stated as blocks, and the treatments as the factor. Separate univariate ANOVAS were conducted when a significant effect was found in the MANOVA (Scheiner, 1993).

The TA levels in *B. suaveolens* damaged by 24 hr feeding by *P. euryanassa* also were assessed 15 d after damage. The procedures were carried out as above, using one additional genet, and the leaves were harvested after 15 d instead of immediately after the 24 hr feeding period. To test whether or not *B. suaveolens* has leaf compensatory growth after damage, we estimated the leaf areas at beginning and end of the experiment (see below), and calculated the total leaf area for the damaged plants as the sum of the leaf area at final harvest plus the leaf area removed by larvae. We calculated the ratio between the total leaf area for damaged plants at final harvest and the leaf area of undamaged plants at final harvest, and we compared the ratios with a single sample *t*-test with a hypothesized mean of one. If the result was not significantly different from one, there is compensatory growth (Belsky, 1986).

Analysis Chemical extraction and TA analysis by GC-MS and GC-FID were carried out according Freitas et al. (1996). Before both GC analyses, samples were derivatized with *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (Acros Organics™) for 30 min at 75°C to prevent the pyrolysis of scopolamine and atropine. Only the four most abundant TAs were quantified by GC-FID using sparteine (Acros Organics™) as internal standard.

Leaf area was estimated from leaf length. Digitalized images of leaves of non-experimental undamaged and damaged plants were used initially to calculate the leaf areas with the software Area Measure 1.00© (1995–97 MYKA Lab.). These areas were used to establish the equation that relates leaf length (cm) to leaf area (mm^2) in undamaged leaves ($ln\ leaf\ area = 0.792\ ln\ leaf\ length + 2.268$, N=51, r^2=0.951, $F_{1,49}$=942.807, P<0.001) and to the area of the half distal portion of the leaves for the damaged leaves ($ln\ leaf\ area = 0.791\ ln\ leaf\ length + 2.376$, N=51, r^2=0.942, $F_{1,49}$=597.672, P<0.001). The first equation was used to assess the leaf area in undamaged and damaged leaves at the beginning and end of the experiment, since after 15 d all leaves had similar geometry. The second equation was used to assess the area of leaf tissue removed by *P. euryanassa* during the 24 hr feeding bout.

Results and Discussion

When considering all expanded leaves, the percentage (mean±SE) of leaf area removed by the larvae during the 24 hr feeding period was 16.7±1.1% in the 24 hr experiment, and 18.7±1.9% in the 15 d experiment. These data did not differ significantly (arc sin transformed data, *t*-test, t=−0.833, df=25, P=0.413) showing that similar damage by larvae was inflicted in both experiments.

Scopolamine and 3-hydroxy-6-tigloyloxytropane were the most abundant TAs found in both undamaged and *P. euryanassa* damaged plants (Fig. 1). The concentration of TAs in damaged plants decreased significantly (around 75%) after 24 hr when compared to undamaged controls (MANOVA, *Wilk's lambda*=0.284, $F_{4,9}$=5.680, P=0.015).

The concentration of scopolamine, 6-hydroxy-atropine, and 3-hydroxy-6-tigloyloxytropane decreased significantly after damage, but atropine was not affected by damage (Fig. 1a). After 15 days, the concentration of TAs between damaged and undamaged plants was not significantly different (MANOVA, *Wilk's lambda*=0.866, $F_{4,10}$=0.390, P=0.813, Fig. 1b).

Brugmansia suaveolens leaf area before the experiment did not differ significantly between treatments (undamaged plants: 401±32; damaged plants: 409±34 mm^2; paired t-test, t=−0.591, df=13, P=0.565). The total area after the 15 day experiment for damaged plants was 785±88 mm^2 and that of undamaged plants at final harvest was 672±77 mm^2. The ratio between the two variables was 1.3±0.1, which did not differ significantly from 1.0 (single t-test, t=1.946, df=13, P=0.074), thus indicating leaf compensatory growth.

The lethal plant defense paradox postulates that the maintenance of defensive compounds at constitutive or induced levels, when plants are attacked by specialists, would be non-adaptative, since they are costly, and not effective against these herbivores (Fordyce, 2001). When *B. suaveolens* was damaged by larvae of the specialist *P. euryanassa* during 24 hr, TA concentration decreases. This plastic response seems to solve part of the paradox. However, when plants were no longer under attack during 15 days, the levels of TAs recovered to their constitutive levels. This recovery may occur since once the attack has stopped, the maintenance of only low levels of defense would be harmful to the plant since TAs may protect it against generalists (Alves et al., 2007). Furthermore, by 15 days after attack, the plants had compensated for loss of tissue due to herbivory. Whether compensatory growth occurs at the expense of chemical defenses (as proposed by Van Der Meijden et al., 1988) or at the expense of roots or other tissues is an open question. Although *B. suaveolens* decreases the TA content and grows when attacked by a specialist, responses to generalists or other specialists are not known. Alternative defense strategies such as the biosynthesis of non sequestrable defensive compounds such as proteins inhibitors, which are not used as defensive compounds by specialists, or attraction of natural enemies also can be used by *B. suaveolens* against both kind of herbivores, and they remain to be investigated.

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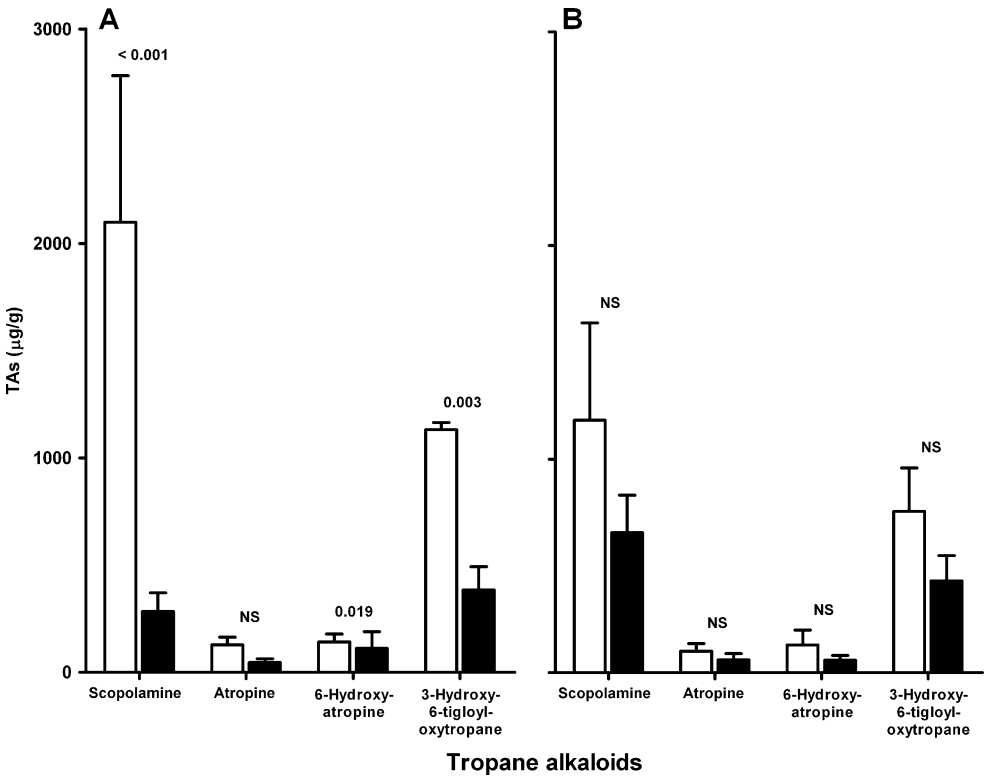


Fig. 1 TA concentrations (µg/g, mean±SE) after 24 hr (**a**) and 15 days (**b**) in leaves of *Brugmansia suaveolens* plants submitted to the treatments: (i) undamage (control=*white bars*) and (ii) damage by larvae of *Placidina euryanassa* (*black bars*). The values above the bars indicate the P value in a separate univariate ANOVA for comparison between undamaged and damaged plants within each TA. For the 24 hr experiment, 13 replicates were used, for 15 days, 14 replicates

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RAPID COMMUNICATION

A Comparison of Sample Preparation Techniques for Quantifying Iridoid Glycosides Sequestered by Lepidopteran Larvae

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Abstract This study compared different methods of tissue preparation for extraction of iridoid glycosides sequestered by three species of lepidopteran larvae. *Junonia coenia* is a specialist on plant species that produce iridoid glycosides, while the arctiids *Estigmene acrea* and *Spilosoma congrua* are both polyphagous and will eat plants that produce iridoid glycosides. Larvae of all three species were reared on leaves of *Plantago lanceolata*, which produces two primary iridoid glycosides, aucubin and catalpol. Three methods of preparing the specimens before extraction in methanol were compared in all three species: 1) larvae were flash-frozen in liquid nitrogen, 2) larvae were macerated fresh in boiling methanol, or 3) larvae were macerated fresh in room temperature methanol. A set of *J. coenia* larvae was oven-dried before maceration as an additional treatment for this species only. *Junonia coenia* sequestered the most iridoid glycosides, while *E. acrea* sequestered the least, and *S. congrua* was intermediate. *Estigmene acrea* was poor at sequestering catalpol. Tissue preparation method only significantly influenced iridoid glycoside recovery from *S. congrua*, with maceration in room-temperature methanol being the most effective of the three methods. This study shows that treatment of insects prior to iridoid glycoside extraction can influence recovery of the compounds, and that the effects of treatment may vary among different species.

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Key Words Arctiidae · Common Buckeye · *Estigmene acrea* · Iridoid glycoside · *Junonia coenia* · Nymphalidae · *Plantago* · *Spilosoma congrua* · Tiger moth

Introduction

An accurate measurement of insect or plant defensive chemistry is necessary for answering a variety of questions in ecology and evolution. The accuracy of measurements can depend on the instrumentation used to measure the chemistry and the methods used to prepare the sample. In this study, we compared three methods of sample preparation for extraction of iridoid glycosides (IGs) from insect species that have the ability to sequester ingested IGs, thus using them for their own chemical defense (Bowers, 1991; Opitz and Müller, 2009).

Iridoid glycosides are polar molecules that are highly soluble in hydrophilic solvents, including water and methanol (Suomi et al., 2000, 2001). These solvents are typically used to recover IGs and other polar compounds from the tissue matrix. Glycosidase enzymes present in the tissues can catalyze glycoside degradation as the sample is prepared for extraction. Insect samples usually are freeze-dried before extraction, or extracted fresh, whereas plant samples may be oven-dried or freeze-dried (e.g., Jamieson and Bowers, 2010). In most studies, insect samples have been frozen in a freezer, although liquid nitrogen also has been used. In one comparison, more IGs were recovered from *Melitaea cinxia* (Nymphalidae) larvae placed in a freezer than from larvae frozen with liquid nitrogen (Suomi et al., 2001). Another potential method to delay or prevent enzymatic breakdown is to boil the sample in order to denature glycosidases before extraction.

We compared three methods of sample preparation before extracting IGs with methanol from three species of lepidopteran larvae that differ in their ability to sequester these compounds (Lampert and Bowers, 2010). *Junonia coenia* Hübner (Common Buckeye) (Nymphalidae) is oligophagous and specializes on plants that produce IGs (Bowers and Stamp, 1997). Larvae exhibit aposematic black and orange coloring and sequester high concentrations of IGs, as much as 20–25% dry weight (Bowers and Stamp, 1997). *Junonia coenia* larvae were contrasted with two polyphagous members of the Arctiidae, *Spilosoma congrua* (Walker) (Agreeable Tiger Moth) and *Estigmene acrea* (Drury) (Saltmarsh Caterpillar). Both of these arctiid species graze among a variety of plant species as larvae and readily consume species that produce IGs (Robinson et al., 2002). Previous work has demonstrated that *S. congrua* sequesters moderate concentrations of IGs, and that *E. acrea* sequesters much smaller amounts (Lampert and Bowers, 2010).

Each species was reared on *Plantago lanceolata* L. (Plantaginaceae), a host plant species that produces aucubin and catalpol, two IGs particularly well studied in association with sequestration and unpalatability (Opitz and Müller, 2009). The amount of IGs recovered was compared among three methods of preparing the insect for extraction: 1) larvae were flash-frozen in liquid nitrogen; 2) larvae were macerated fresh in boiling methanol; or 3) larvae were macerated fresh in room temperature methanol. A fourth method of oven-drying larvae before extraction in methanol was also done for *J. coenia* alone.

Methods and Materials

Larvae were reared in groups of 10 in 500 ml plastic containers on freshly excised *Plantago lanceolata* leaves in a growth chamber (25°C, 16:8 h L:D). Larvae were weighed to the nearest 0.1 mg and processed for IG extraction immediately upon reaching the final instar (5th for *J. coenia*, 6th for *S. congrua* and *E. acrea*). The final instars were handled in three ways (four ways for *J. coenia)* before a standard extraction in methanol. One set of larvae of each species was flash-frozen using liquid nitrogen, pulverized with a mortar and pestle, and extracted in 5 ml room temperature (~20°C) 95% methanol for 48 hr. A second set of larvae of each species was anesthetized with carbon dioxide, then macerated fresh with sand in 5 ml room temperature 95% methanol, and extracted for 48 hr. A final set of larvae of each species was anesthetized with carbon dioxide, then macerated fresh with sand in 5 ml boiling (~65°C) methanol, and extracted at room temperature for 48 hr. An additional fourth treatment was included for *J. coenia.* The larvae were frozen in a freezer at −20°C, oven-dried for 72 hr at 55°C, then macerated with sand in 5 ml room temperature methanol, and extracted for 48 hr. Samples were vortexed at ~8 hr intervals during the 48 hr extraction, and were otherwise undisturbed.

Sample sizes were 10 individual larvae for all extraction procedures. The aim of this study was to compare tissue preparation rather than chromatography methods, and all samples were analyzed by using the same methods for sample preparation and analysis with gas chromatography with a flame-ionization detector (GC-FID) (Bowers and Stamp, 1997). In short, the sample was filtered to remove solid material; the methanol was evaporated; and the internal standard, phenyl-β-D-glucopyranoside (PBG) (Sigma-Aldrich), dissolved in water (0.500 mg per ml), was added. The sample was partitioned between water and ether, the ether layer was discarded, the water was evaporated, and the residue was redissolved in 1 ml methanol. A 100 µl aliquot was evaporated and derivatized with 100 µl Tri-Syl-Z (Pierce Chemical Company, Rockford, IL, USA) in pyridine before injection. Amounts of aucubin and catalpol were measured by using Agilent ChemStation A.03.34 software with pure standards of the compounds. To directly compare the results from the different methods, the dry weight of fresh larvae was estimated by using a separate set of larvae of each species, weighing them fresh, drying them to a constant weight in an oven, and then weighing them dry (N= 25 for each). A conversion factor was calculated and applied to larvae that were extracted fresh to obtain a dry weight for each caterpillar.

A series of Bonferroni-adjusted 1-way analyses of covariance (ANCOVA) were performed for each species to compare the amount (mg/larvae) of aucubin, catalpol, and total IGs among the three (*S. congrua*, *E. acrea*) or four (*J. coenia*) preparation methods, treating caterpillar dry mass as a continuous covariate. A separate set of 1-way analyses of variance (ANOVA) was used to compare the concentration (% dry mass) of aucubin, catalpol, and total IGs among preparation methods. Concentrations were treated as proportions and arcsine square-root transformed prior to analysis. Because only 6 of 30 *E. acrea* individuals contained measurable amounts of catalpol, analyses were done only on amount of aucubin and total IGs for this species. Both amounts and concentrations of IGs were compared among preparation methods using Tukey's Honestly Significant Difference *post-hoc* tests. Tests were conducted separately for each measure for each caterpillar species where appropriate.

Results and Discussion

Junonia coenia sequestered, on average, the largest amounts of IGs, particularly catalpol, compared to the

other species (Table 1). This variation in sequestration ability among species was consistent among all tissue preparation methods, and consistent with a previous study (Lampert and Bowers, 2010).

There was no significant effect of sample preparation method on amount (mg) or concentration of IGs in either *J. coenia* or *E. acrea* ($P>0.05$ for all comparisons, Table 1). Although not compared statistically, in all treatments *Junonia coenia* larvae sequestered larger amounts and higher concentrations of catalpol than aucubin (Table 1). *Estigmene acrea,* in contrast, was poor at sequestering both compounds, and most larvae (24/30) contained no catalpol at all.

Results were quite different for *S. congrua*. Although *S. congrua* larvae were fed from the same batches of *P. lanceolata* leaves as were *J. coenia* and *E. acrea*, they sequestered larger amounts and higher concentrations of aucubin than catalpol. In a previous study (Lampert and Bowers, 2010), *S. congrua* was relatively efficient at sequestering catalpol, although the experimental design and leaf material fed to larvae were different. These differences in results may have been due to differences in the IG content of leaves on which caterpillars were fed.

For *S. congrua,* the amount of total iridoid glycosides (mg) per insect recovered was significantly influenced by sample preparation method ($F=6.170$, $P=0.007$), as were amounts of aucubin ($F=3.411$, $P=0.049$) and catalpol ($F=3.998$, $P=0.031$). The covariate of insect dry mass was not significant in any of the analyses ($P>0.30$ for each analysis). *Post-hoc* comparisons showed that amounts of aucubin extracted after all treatments was the same ($P>0.075$ in all cases). More catalpol and more total iridoid glycosides were obtained with maceration in room temperature methanol compared with boiling methanol (Table 1). Likewise, the concentration of total IGs was significantly influenced by sample preparation method in this species ($F_{2,25}=10.05$, $P=0.001$) (Table 1). Similar results were obtained for concentrations of both aucubin ($F=5.968$, $P=0.007$) and catalpol ($F=6.612$, $P=0.005$). *Post-hoc* comparisons showed that for concentrations of aucubin, catalpol, and total IGs, room temperature methanol resulted in the highest levels of recovery, and this was significant when compared with liquid nitrogen, but not boiling methanol (Table 1).

Secondary compound recovery can be influenced by how the sample is extracted (Suomi et al., 2000, 2001), and the comparison of extractions in different solvents is important. Suomi et al. (2000) compared water to ethanol and methanol as an extraction solvent for aucubin and catalpol from plant tissues, and found water to be more effective for both compounds. Since plant and insect tissues store IGs differently, it is unknown whether water and methanol are equally effective solvents for extraction. In a preliminary study, we did not find significant differences between water and methanol extraction of *J. coenia* iridoid glycosides (unpublished data).

We found that recovery of sequestered IGs using a single detection method (GC-FID) can be influenced by the way the insect sample is handled prior to extraction. Iridoid glycoside recovery was more variable in one insect species than in two others, possibly due to differences in how the species sequester these compounds or the biochemical pathways or enzymes used for metabolism of these ingested compounds. Notably, for *S. congrua,* there was a significant effect of preparation method on IG recovery, and macerating the insect fresh in room-temperature methanol resulted in the highest recovery of IGs. Neither boiling methanol nor flash-freezing, both of which were hypothesized to inhibit enzymatic breakdown of IGs, improved recovery. These

Table 1 Average (±1 SEM) amounts (mg per individual) and percentage of insect dry weights of the iridoid glycosides aucubin and catalpol recovered from 3 caterpillar species

Species	Preparation method	aucubin (mg)	catalpol (mg)	total IGs (mg)	aucubin (%)	catalpol (%)	total IGs (%)
Junonia coenia	liquid nitrogen	1.35±0.15	2.18±0.23	3.53±0.38	6.73±0.47	10.91±0.83	17.64±1.26
	methanol	1.44±0.14	1.97±0.16	3.41±0.30	7.39±0.69	10.11±0.55	17.50±1.00
	boiling methanol	1.07±0.14	1.87±0.21	2.94±0.34	6.05±0.60	10.64±1.03	16.70±1.58
	oven-dried	1.22±0.10	1.44±0.10	2.66±0.18	7.74±0.69	9.13±0.86	16.88±1.47
Spilosoma congrua	liquid nitrogen	1.54±0.19	0.85±0.07ab	2.39±0.21ab	2.15±0.29b	1.17±0.12b	3.32±0.32b
	methanol	1.91±0.14	0.99±0.08a	2.90±0.20a	3.54±0.36a	1.90±0.19a	5.43±0.41a
	boiling methanol	1.29±0.10	0.73±0.03b	2.02±0.10b	2.75±0.24ab	1.55±0.09ab	4.30±0.39ab
Estigmene acrea	liquid nitrogen	0.16±0.10	0.015	0.17±0.099	0.20±0.12	0.005	0.20±0.12
	methanol	0.02±0.015	0.001	0.02±0.015	0.04±0.02	0.002	0.04±0.02
	boiling methanol	0.06±0.03	0.025±0.02	0.07±0.04	0.07±0.03	0.008±0.007	0.08±0.04

Ten individuals of each species, all reared only on *Plantago lanceolata* leaves, were sampled. Lowercase letters indicate significantly ($P<0.05$) different means according to Tukey's Honestly Significant Difference *post-hoc* tests. *Post-hoc* tests were conducted separately for each measure for each species. IG = iridoid glycoside

data suggest that maceration in room-temperature methanol is a suitable initial step in extracting IGs from insect larvae. Future research should compare extraction solvents and instrumentation to determine the most accurate methods to extract and measure iridoid glycosides.

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Changes in Energy Metabolism and Antioxidant Defense Systems During Seed Germination of the Weed Species *Ipomoea triloba* L. and the Responses to Allelochemicals

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Abstract The relationships between changes in energy metabolism and the antioxidant defense system in the weed species *Ipomoea triloba* L. during seed germination and early seedling growth were investigated. The effects of some common allelochemicals on these parameters also were studied. Respiratory activity and the activities of alcohol dehydrogenase, superoxide dismutase, catalase, guaicol peroxidase, ascorbate peroxidase, glutathione reductase, and lipoxygenase were measured. Mitochondrial oxidative phosphorylation resumed shortly after the seed imbibition period, as indicated by considerable KCN-sensitive respiratory activity in embryos of *I. triloba*. The occurrence of superoxide dismutase, catalase, guaicol peroxidase, and lipoxygenase activities in the embryos, along with significant KCN-insensitive respiration, suggest that production of reactive oxygen species (ROS) is initiated as soon as mitochondrial respiration is resumed. All assayed antioxidant enzymes were present in the embryos except ascorbate peroxidase, which appeared only in primary roots. The activities of antioxidant enzymes increased after completion of germination, especially in primary roots. Superoxide dismutase, catalase, and guaicol peroxidase probably were the crucial enzymes involved in the neutralization of ROS, since they had higher levels of activity compared with other enzymes, such as ascorbate peroxidase and glutathione reductase. When seeds were grown in the presence of α-pinene, coumarin, quercetin, and ferulic acid, there was an additional increase in activities of antioxidant enzymes, as well as increases in lipoxygenase activity and KCN-insensitive respiration, suggesting a further increase in ROS generation. The antioxidant defense system of *I. triloba* was not effective in preventing lipid peroxidation caused by α-pinene. The data indicate that during seed germination and initial growth of *I. triloba*, a period when antioxidant enzyme activity increases to counteract the harmful ROS effects produced during mitochondrial metabolism resumption, the presence of allelochemicals, which cause further oxidative stress, may leave the seeds/seedlings more vulnerable to cellular dysfunction and cell death.

Key Words Antioxidant enzymes · *Ipomoea triloba* L. · Germination · Allelochemical · Respiration

Introduction

Seed germination is a critical step in the plant life cycle. When quiescent dry seeds imbibe water, their oxygen uptake increases, and mitochondrial energy metabolism becomes active, allowing for macromolecule biosynthesis and solute transport that are required to complete germination. Reactivation of mitochondrial metabolism also provides an important source of reactive oxygen species (ROS). Under normoxic conditions, approximately 2–3% of the oxygen used by mitochondria can be converted into superoxide and H_2O_2 (Puntarullo et al., 1988).

The toxicity of ROS, which causes oxidative damage to cell macromolecules, is well documented (Gidrol et al., 1994; Bailly et al., 1996; McDonald, 1999). There now is evidence that ROS also play normal physiological roles, acting as signaling molecules in many processes, including seed germination and dormancy alleviation (Kwak et al., 2006; Bailly et al., 2008; Oracz et al., 2009). The shift from

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a normal role in cell signaling to a toxic role with deleterious effects is probably related to changes in ROS homeostasis that result from a shift in the balance between ROS-producing and ROS-scavenging processes. Plants contain soluble antioxidant compounds, such as ascorbic acid, vitamin E, and glutathione (Noctor and Foyer, 1998; De Tullio and Arrigoni, 2003), and a battery of enzymes that can efficiently destroy superoxide radicals and hydrogen peroxide, including superoxide dismutase, catalase, peroxidases, thioredoxin system enzymes, and ascorbate glutathione cycle enzymes (Navrot et al., 2007).

Most studies of antioxidant enzyme activities during seed germination have evaluated changes caused by aging or abiotic stress conditions such as drying (Bailly et al., 2001), high temperature, and high humidity (Gidrol et al., 1994; Bailly et al., 2002). There are few studies that are concerned with the relationships between mitochondrial respiratory activity and the antioxidant defense system during seed germination and initial seedling growth, particularly in weed species.

Common weeds are relatively difficult to control in field crops due to high seed production and their long viability. *Ipomoea triloba* L. (syn *Ipomoea grandifolia* (Dammer) O'Don), a tropical and subtropical weed, commonly known as morning glory (Holm et al., 1997) is a dicot of the family *Convolvulaceae*. It causes yield losses of several crops, particularly soybean (*Glycine max* L.) (Holm et al., 1997). In addition to intrinsic and extrinsic factors, (Boyd and Acker, 2004), allelochemicals in the soil also can affect seed germination by altering an essential biochemical or physiological process, such as mitochondrial energy metabolism (Ishii-Iwamoto et al., 2006).

Numerous allelochemicals of the phenolic, flavonoid, and terpenoid classes have been suggested as the compounds implicated in the plant growth suppression, including that of weed species (Vaughn and Spencer, 1993; Dudai et al., 1999; Reigosa et al., 1999; Duke et al., 2000; Singh et al., 2006; Batish et al., 2004, 2007; Chon and Kim, 2004; Kohli et al., 2006). When these compounds decrease plant growth, the phenomenon is considered to be a biotic stress termed "allelochemical stress" (Cruz-Ortega et al., 2002; Romero-Romero et al., 2002).

Perturbation of the pathways that generate ROS, such as the mitochondrial respiratory chain or the activities of antioxidant enzymes, could be a mechanism underlying allelochemical stress during seed germination and initial seedling growth. It has been demonstrated, for example, that the monoterpene α-pinene induces oxidative stress in seedlings through a direct action on mitochondrial metabolism (Abrahim et al., 2003a, b; Ishii-Iwamoto et al., 2006). In mitochondria isolated from primary roots of maize (*Zea mays*), α-pinene uncouples electron transport from ATP synthesis, decreases the membrane potential, and inhibits the alternative oxidase pathways (Abrahim et al., 2003a). Consistent with these effects, in intact primary roots, α-pinene inhibits KCN-sensitive respiration, stimulates KCN-insensitive respiration and lipoxygenase activity, and increases the level of conjugated dienes (Ishii-Iwamoto et al., 2006). Singh et al. (2006) also reported that α-pinene induces oxidative stress in roots of *Cassia occidentalis*. It increases the levels of malondialdehyde, proline, and hydrogen peroxide, and it elevates the activities of antioxidant enzymes. Oxidative stress induction has been suggested to be the mode of actions of other allelochemicals, including coumarin (Abenavoli et al., 2006; Pergo et al., 2008), catechin (Bais et al., 2003), and 2-benzoxazolinone (Batish et al., 2006).

It is plausible that the ability of seeds to germinate might be related, in part at least, to their capacity to regulate the levels of ROS that are generated during resumption of mitochondrial oxygen consumption, and also to neutralize the pro-oxidant activities of allelochemicals present in the medium.

Thus, this study was undertaken to investigate the relationship between the resumption of respiratory activity and the kinetics of the changes in the activities of the main antioxidant enzymes during seed germination in the weed species, *Ipomoea triloba* L., and the influence of phytotoxic allelochemicals on these processes. Fourteen different natural plant compounds were screened for activity on *Ipomoea triloba* L. They represent the phenolic and terpenoid classes, namely, vanillic acid, *p*-coumaric acid, protocatechuic acid, ferulic acid, caffeic acid, caffeine, aconitic acid, quercetin, rutin, coumarin, eucalyptol, camphor, α-pinene, and limonene.

The parameters of mitochondrial respiratory activity, the activities of alcohol dehydrogenase (ADH), and the activity of antioxidant enzymes implicated in ROS balance were measured. The enzyme activities measured include superoxide dismutase (SOD), catalase (CAT), guaicol peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR). Membrane damage by means of lipid peroxidation was evaluated by measurements of malondialdehyde (MDA) content and lipoxygenase activity (LOX). The content of oxidized (GSSG) and reduced glutathione (GSH) in allelochemical-treated seedlings also was examined.

Methods and Materials

Reagents KCN, NAD^+, ascorbate, GSSG, NADPH, linolenic acid, 2-thiobarbituric acid, vanillic acid, *p*-coumaric acid, protocatechuic acid, ferulic acid, caffeic acid, caffeine, aconitic acid, quercetin, rutin, coumarin, eucalyptol, camphor, α-pinene, and limonene were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Seed Germination and Initial Growth Seeds of *Ipomoea triloba* L., were purchased from a commercial supplier (Cosmos Agrícola Produtos e Serviços Rurais Ltda, Brazil). Seeds were placed on double sheets of germination paper in plastic germination boxes (110×110 mm) and moistened with 10 ml of distilled water or 500 μM allelochemicals. Vanillic acid, p-coumaric acid, protocatechuic acid, ferulic acid, caffeic acid, caffeine, aconitic acid, quercetin, and rutin were dissolved in distilled water. Coumarin, eucalyptol, camphor, α-pinene, and limonene were dissolved in a 0.1% dimethylsulfoxide (DMSO) solution. Controls were performed to exclude the interference of dimethylsulfoxide. Each treatment was applied to three plates (replicates), and each replicate consisted of 60 seeds distributed among the germination boxes. Boxes were placed in a germination chamber programmed for a 12-hr photoperiod (photon flux density of approximately 230 μmol m^{-2} s^{-1}) at 30°C. Seeds that had germinated at 24, 48, and 72 hr were selected for growth tests. The hypocotyl and the primary roots were excised for measurements of their length and fresh weight. Dry weight was evaluated after incubation of the fresh materials at 80ºC for 24 hr.

Mean germination time was calculated by means of Eq. 1 (Labouriau and Osborn, 1984):

$$\bar{t} = \sum n_i t_i \Big/ \sum n_i \quad (1)$$

$\bar{t}$ Mean germination time
n_i Number of germinated seeds between the times $t_{i\text{-}1}$ and t_i

The speed of germination was calculated by means of Eq. 2 (Chiapuasis et al., 1997):

$$S = (N_1/T_1) + (N_2 - N_1) \times 1/2 + (N_3 - N_2) \times 1/3 + \ldots (N_n - N_{n-1}) \times 1/n \quad (2)$$

AS Speed of germination
N Proportion of germinated seeds obtained in the first (T_1), second (T_2), third (T_3)…, (n-1) hr.

The speed of accumulated germination was calculated by means of Eq. 3 (Chiapuasis et al., 1997):

$$AS = (N_1/T_1) + (N_2/T_2) + (N_3/T_3) + \ldots (N_n/n) \quad (3)$$

S Speed of accumulated germination
N Proportion of germinated seeds on time (T_1), time (T_2), time (T_3)…, hr.

For measurements of respiratory activity and antioxidant enzyme activities, the embryos, cotyledons, and primary roots were removed from imbibed seeds or seedlings that had been incubated for 12, 24, 36, and 48 hr after the onset of seed imbibition. The data collected at 12 hr refer to embryo tissues. After at least 24 hr, the germinated seeds were selected. The primary roots were excised, and the cotyledons were separated from the remaining seeds.

Measurements of Respiratory Activity Embryos and cotyledons were carefully separated after removal of seed coats. Primary roots were cut into segments 5–10 mm long as measured from the growth apex, and then weighed. After weighing, samples were placed immediately in the oxygen electrode vessel with 2 ml of nutrient solution (pH 5.8) containing 2 mM $Ca(NO_3)_2$, 2 mM KNO_3, 27 μM $FeCl_3$, 0.43 mM NH_4Cl, 0.75 mM $MgSO_4$, and 20 μM NaH_2PO_4 (Larkin, 1987). To estimate the contributions of mitochondrial cytochrome oxidase (COX; KCN-sensitive respiration), alternative oxidase (AOX), and the extramitochondrial oxidases (KCN-insensitive respiration) to the overall O_2 uptake, 200 μM potassium cyanide (KCN) was added to the reaction medium. Oxygen uptake rates were calculated from the polarographic records, assuming an initial concentration of dissolved oxygen of 240 μM at 25°C (Estabrook, 1967).

Preparation of Enzyme Extracts Embryos, cotyledons, and primary roots were removed from imbibed seeds or seedlings, and samples of approximately 200 mg fresh weight of each tissue were transferred to a mortar and thoroughly mixed with 2.0–4.0 ml of extraction medium containing: for alcohol dehydrogenase, 50 mM Tris–HCl (pH 7.4), 1.0 mM EDTA, and 2.0 mM dithiothreitol (DTT); for peroxidase and catalase, 67 mM K-phosphate (pH 7.0) and 2% PVP (w/v); for ascorbate peroxidase and glutathione reductase, 50 mM K-phosphate (pH 7.0), 1 mM EDTA, 1 mM ascorbate, and 2% PVP (w/v); for superoxide dismutase, 50 mM K-phosphate (pH 7.8), 1.0 mM EDTA, 1% PVP (w/v), and 0.1% Triton X-100 (v/v); and for lipoxygenase, 50 mM K-phosphate (pH 7.0) and 0.1% Triton X-100 (v/v). Extracts were centrifuged for 20 min at 4000 rpm and 4°C. The supernatant was decanted and used as the enzyme source. The protein concentration of each enzyme extract was determined according to Lowry et al. (1951). Bovine serum albumin was used as a standard.

Determination of Enzyme Activities Alcohol dehydrogenase activity was measured in a medium containing 50 mM Tris–HCl (pH 7.4), 1.0 mM NAD^+, 120 μM n-propanol, and 0.1–0.3 mg protein of enzyme extract (Lee, 1982). The rate of NAD^+ reduction was monitored at 340 nm.

Peroxidase activity was measured in a medium containing 25 mM K-phosphate (pH 6.8), 10 mM H_2O_2, 2.6 mM

guaiacol, and 0.1–0.4 mg protein of enzyme extract. Tetraguaicol formation (ε, 25.5 mM^{-1} cm^{-1}) was measured at 470 nm (Pütter, 1974).

Catalase activity was measured in a medium containing 67 mM K-phosphate (pH 7.0), 10 mM H_2O_2, and 0.1–0.4 mg protein of enzyme extract. The consumption of H_2O_2 was monitored at 240 nm (ε, 0.036 mM^{-1} cm^{-1}) (Aebi, 1984).

Ascorbate peroxidase activity was measured in a medium containing 50 mM K-phosphate (pH 7.0), 0.5 mM ascorbate, 2 mM H_2O_2, and 0.1–0.4 mg protein of enzyme extract. Ascorbate oxidation was measured at 290 nm (ε, 2.8 mM^{-1} cm^{-1}) (Nakano and Asada, 1981).

Glutathione reductase activity was measured in a medium containing 50 mM K-phosphate (pH 8.0), 2 mM EDTA, 0.5 mM GSSG, 0.15 mM NADPH, and 0.1–0.4 mg protein of enzyme extract. The rate of NADPH oxidation was monitored at 340 nm (ε, 6.2 mM^{-1} cm^{-1}) (Foyer and Halliwell, 1976).

Superoxide dismutase activity was measured according to Giannopolitis and Ries (1977). The medium contained 50 mM K-phosphate (pH 7.8) 6.5 mM methionine, 150 μM nitro blue tetrazolium NBT, 4 μM riboflavin, and 0.02–0.1 mg protein of enzyme extract. The reaction was started by switching on a light (20 W) and illuminating the medium for 20 min at 30ºC. One unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of the (NBT) photoreduction rate read at 560 nm, and the results were expressed as U of SOD×mg $protein^{-1}$.

Lipoxygenase activity was measured polarographically with a Clark-type oxygen electrode in a medium containing 200 mM K-phosphate (pH 7.0), linolenic acid (1.5 mM final concentration, dissolved in Tween 20), and 0.1–3.0 mg protein of enzyme extract (Siedow and Girvin, 1980). The enzyme activity was expressed as μmol O_2 consumed×min^{-1}×mg $protein^{-1}$.

Determination of MDA, Oxidized Glutathione (GSSG) and Reduced Glutathione (GSH) Primary roots were excised from seedlings grown for 48 hr in the absence or presence of 500 μM of each allelochemical. The level of lipid peroxidation was measured relative to the thiobarbituric acid reacting substance, malondialdehyde (MDA) (Heath and Packer, 1968). Approximately 0.6 g fresh weight of roots were extracted with 3.0 ml of 96% (v/v) ethanol. An equal volume of 20% TCA containing 0.5% TBA (w/v) was added to the homogenate. The mixture was heated at 95°C for 30 min. After centrifuging, absorbance of the supernatant at 532 nm was read. The non-specific absorbance at 600 nm was subtracted. The concentration of MDA was calculated by using its extinction coefficient of 155 mM^{-1} cm^{-1}. For a glutathione content measurement, samples of primary roots (0.6 g fresh weight) were ground in liquid nitrogen. The frozen powder was homogenized in 5% sulfosalicylic acid and centrifuged at 10,000×g for 15 min at 4°C. Total glutathione was determined in a medium containing 0.5 M potassium phosphate buffer (pH 7.5), 50 mM sodium phosphate buffer (pH 7.5), 5 mM EDTA, 1.0 mM dithionitrobenzoic acid (DTNB), 2 Uml^{-1} glutathione reductase, and 0.17 mM NADPH (Smith, 1985). After 10 min, the absorbance at 415 nm was read. For measurement of the oxidized glutathione, the enzyme extract was previously treated with 1.7 mM β-vinylpyridine dissolved in 2% Tween 20 (v/v) for 1 hr at room temperature. The concentration of GSSG and GSH was calculated using the extinction coefficient of 13.6 mM^{-1} cm^{-1}.

Statistical Analysis The data shown in the graphs and tables are expressed as means ± standard errors of the mean (SEM) of independent preparations. Data were analyzed using the Student's *t*-test or variance analysis (ANOVA). Significant differences between means were identified by Duncan's multiple range test. $P \leq 0.05$ was adopted as the minimum criterion of significance. Statistical analyses were performed using the Statistica™ software package.

Results

Background Information on the Germination and Growth Behavior of Ipomoea triloba Seeds of *I. triloba* started to germinate at nearly 24 hr after imbibition, achieving a maximum germination percentage of 33% at 72 hr (Fig. 1a). The growth of primary roots and hypocotyls from the germinated seeds, in contrast, increased progressively, as indicated by their lengths (Fig. 1b). Between 24 and 72 hr of incubation, primary root and hypocotyl lengths increased 3.9-fold and 10.3-fold, respectively. Fresh weights (Fig. 1c) and dry weights (Fig. 1d) of primary roots and hypocotyls also increased progressively. After 72 hr of incubation, the first leaves were visible, indicating that photosynthesis had started to contribute to seedling energy metabolism. Thus, the subsequent experiments were performed with seedlings grown for a maximum of 48 hr, assuring that the contribution of mitochondrial respiration to the energy metabolism of the seedlings was predominant.

Respiratory Activity and ADH Activity in Embryos, Cotyledons and Primary Roots There was considerable respiratory activity in embryos during the seed imbibition period (12 hr of incubation) (Fig. 2a). After germination completion (24 hr), the activity in cotyledons was nearly 2-fold higher, and remained elevated until 36 hr. At 48 hr, it had

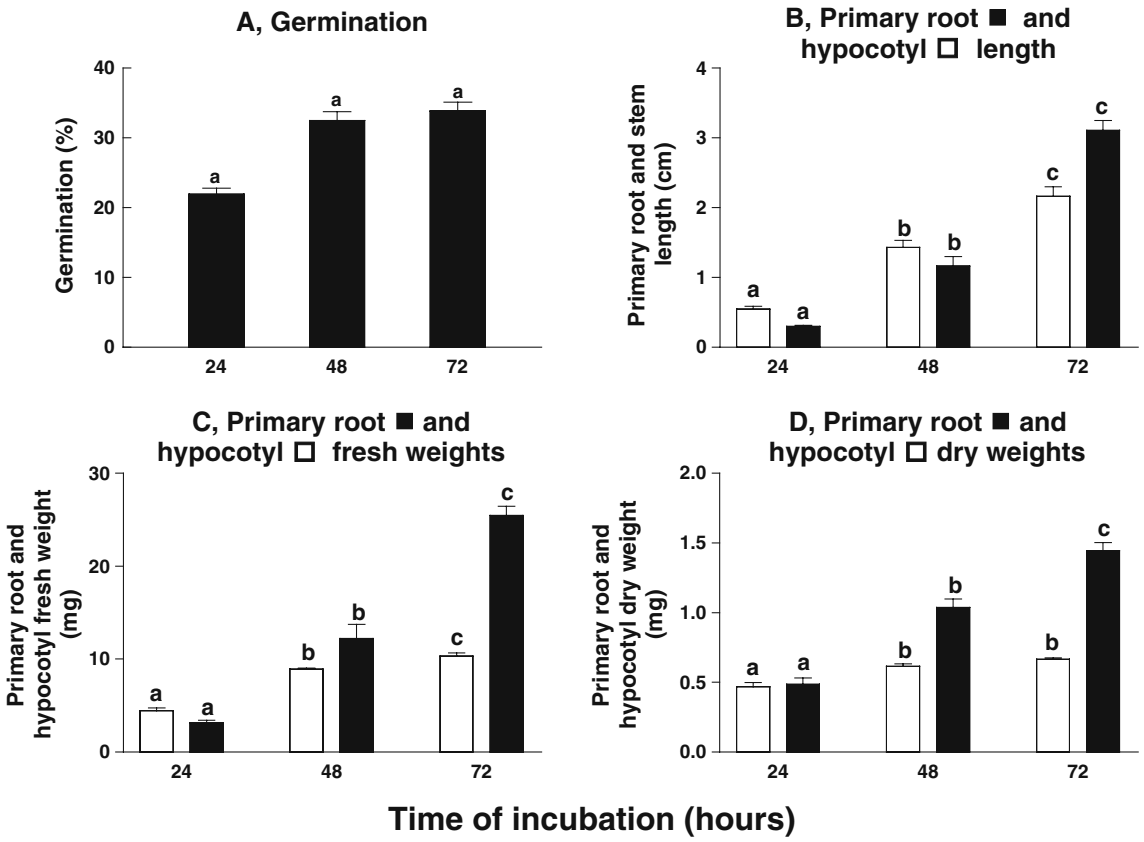


Fig. 1 Germination (**a**), primary root and hypocotyl lengths (**b**), root and hypocotyl fresh weights (**c**), root and hypocotyls dry weights (**d**) of *Ipomoea triloba.* The values are the means ± SE (*N*=4–5). *White bars* in panels **b**, **c**, and **d** are the values of hypocotyls and black bars are the values of primary roots. Different letters within each parameter and in the same tissue (hypocotyls or primary root) indicate significant differences according to ANOVA with Duncan's multiple range test at 5% level significance

decreased by nearly 50%. The relative contribution of KCN-insensitive respiration to the overall respiration varied from 22% to 34.3%. The primary roots presented a lower total respiratory activity when compared with their respective cotyledons (Fig. 2b). There was a gradual decrease during the growth period, and at 48 hr it was reduced by 40.4% compared to the value found at 24 hr. The relative contribution of KCN-insensitive respiration to the overall respiration in primary roots was, in general, higher than that found in cotyledons. It increased to 32.5% and 60% at 24 and 48 hr, respectively.

The activity of ADH was higher in embryos (12 hr) than in cotyledons at 24 hr (Fig. 2c). ADH activity in the primary roots at 24 hr was similar to that in the embryo (Fig. 2d). From 24 to 48 hr of incubation, there was a progressive decline of enzyme activity in both cotyledons and primary roots (Fig. 2c, d).

Changes in Enzyme Activities in Germinating Seeds SOD activity was detected in embryos during the seed imbibition period (Fig. 3a). After germination (24 hr), SOD activity in the cotyledon was not different from that in the embryo. On the other hand, SOD activity in the primary roots was nearly two-fold higher. During the subsequent growth period, enzyme activity did not change significantly in either the cotyledons or the primary roots.

The pattern of changes in CAT (Fig. 3b) and POD (Fig. 3c) activities were similar: the activity in cotyledons did not differ from that in embryos and did not change during the subsequent growth periods. In primary roots, however, the activity of both enzymes increased. At 48 hr, the activities of CAT and POD increased nearly 2- and 3-fold, respectively, relative to the corresponding values found at 24 hr.

As shown in Fig. 3d, the activity of GR was very low in *I. triloba* in all tissues throughout the experimental growth period.

APX was not detected during seed imbibition (Fig. 3e), and in cotyledons significant activity was found only after 48 hr of incubation. Enzyme activity appeared, however, in primary roots after the completion of germination (24 hr) and increased during the subsequent growth period. At 48 hr, the activity was 4-fold higher than the values found at 24 hr.

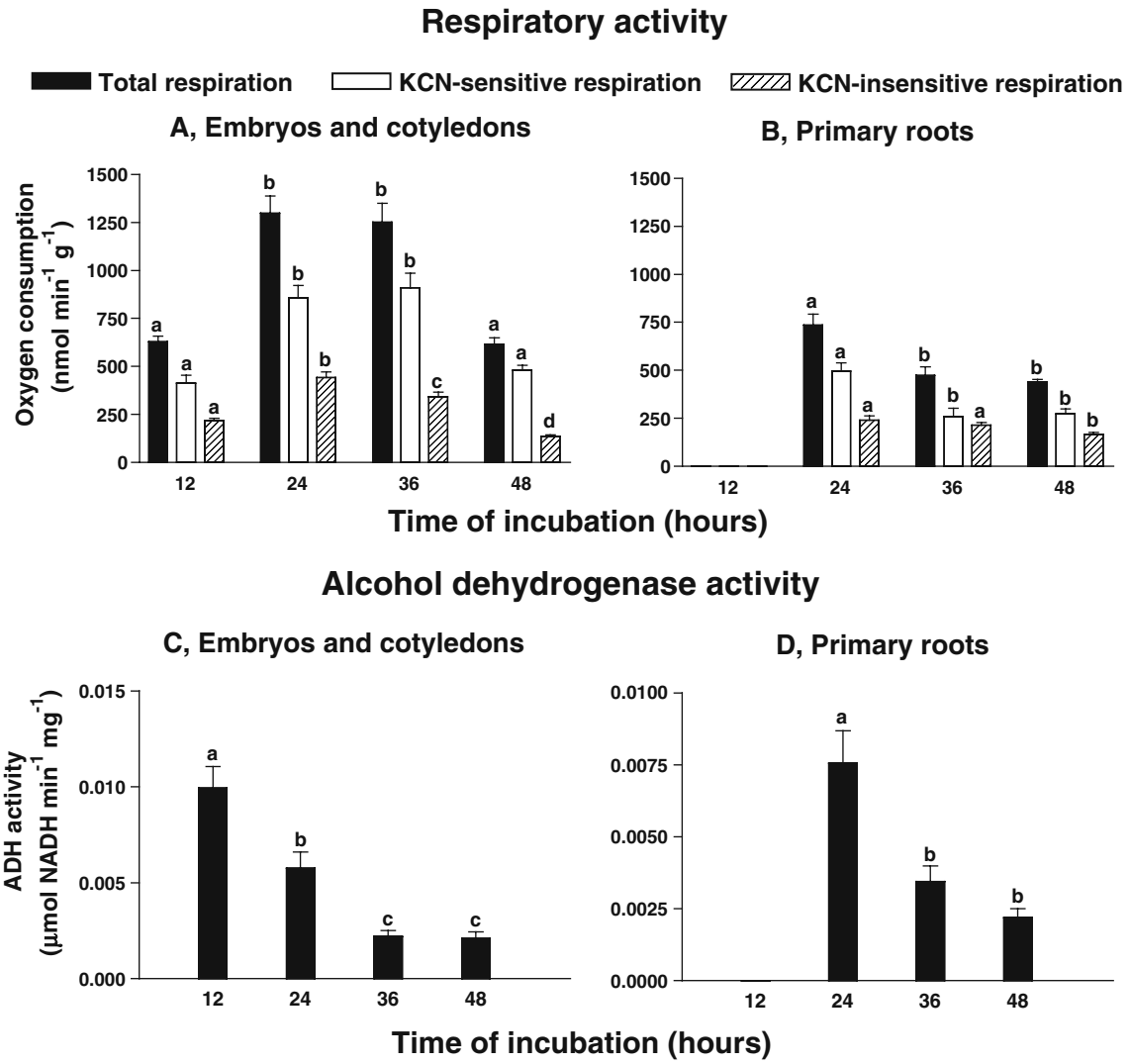


Fig. 2 Time-course of respiratory activity (**a**, **b**) and alcohol dehydrogenase (ADH) activity (**c**, **d**) of embryos, cotyledons (**a**, **c**) and primary roots (**b**, **d**) of *Ipomoea triloba*. Total respiration: oxygen consumption in the absence of KCN; KCN-insensitive respiration: oxygen consumption in the presence of KCN; KCN-sensitive respiration: difference between total respiration and KCN-insensitive respiration. The values are the means ± SE (N=4 for respiratory activity and N=5 for ADH). Different letters within each parameter indicate significant differences according to ANOVA with Duncan's multiple range test at 5% level significance

The activity of LOX (Fig. 3f) was relatively negligible in embryos of *I. triloba*, but there was an accentuated increase after the completion of seed germination. At 24 hr, the activity of this enzyme was higher in cotyledons than in primary roots. The activity did not vary in cotyledons during the growth period, but it increased in primary roots by 3.7-fold at 36 hr and by 4.1-fold at 48 hr relative to the value recorded at 24 hr.

Effects of Fourteen Allelochemicals on Germination and Growth of Ipomoea triloba L. None of the assayed allelochemicals caused significant modification in germination indices of *I. triloba* (Table 1). However, the growth of the resulting seedlings was reduced, especially the primary root growth (Table 2). At 48 hr, root length was reduced by vanillic acid (−27%), coumaric acid (−33%), ferulic acid (−31%), caffeine (−25%), quercetin (−22%), eucalyptol (−16%), camphor (−26%), α-pinene (−22%), and limonene (−17%). Coumarin was more active; it reduced root length by 62% and also affected hypocotyl length (−47%). Hypocotyl length also was reduced by camphor (−28%). A parallel decrease in the fresh weight of primary roots was found for vanillic acid, coumaric acid, ferulic acid, quercetin, and α-pinene. Rutin did not cause any change in primary roots length, but it reduced the fresh weight of primary roots. None of the assayed allelochemicals modified the fresh weights of hypocotyls. For subsequent experiments, we selected among the active compounds a representative of the

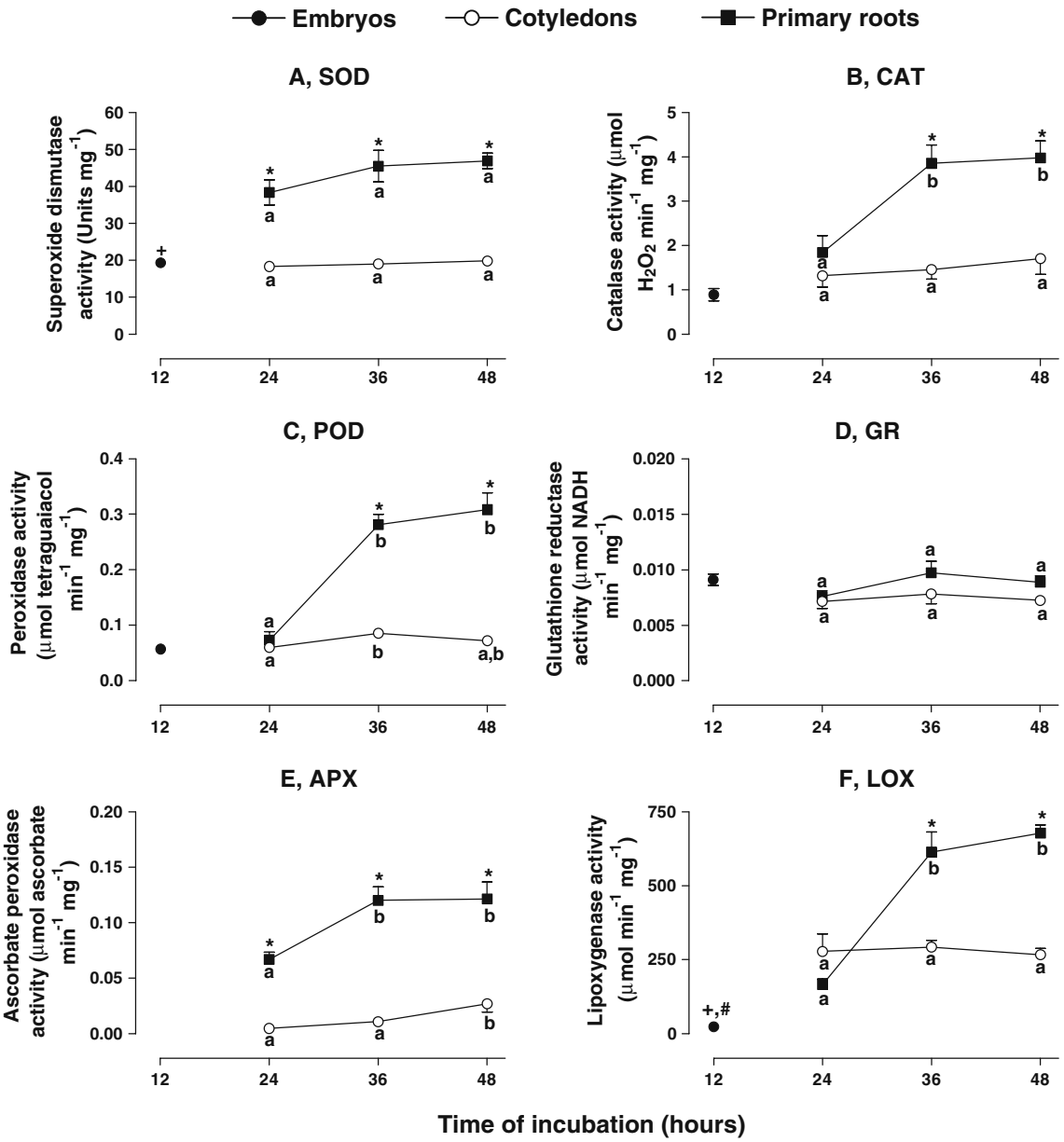


Fig. 3 Time-course of superoxide dismutase (**a**), catalase (**b**), peroxidase (**c**), glutathione reductase (**d**), ascorbate peroxidase (**e**) and lipoxygenase (**f**) activities in embryos, cotyledons and primary roots of *I. triloba*. Each data point is the mean ± SE (N=4–7). Significant differences between means were identified by ANOVA with Duncan's multiple range test (P<0.05). Different letters indicates mean values of enzymatic activities in embryos and cotyledons or in primary roots that are significantly different; (*) Values of primary roots differing statistically from values of cotyledons at the same experimental time; (+) Values of embryo (12 hr) differing statistically from values of primary roots at 24 hr; (#) Values of embryo (12 hr) differing statistically from values of cotyledons at 24 hr

terpene (α-pinene), phenolic (ferulic acid), and flavonoid (quercetin) class, and coumarin, the most active compound. The respiratory activity, the enzyme activities, and the content of MDA and glutathione were measured in primary roots from seedlings grown for 48 hr in the presence of these selected allelochemicals.

Effects of α-Pinene, Coumarin, Quercetin and Ferulic Acid on Root Apex Respiration, Enzyme Activities and Parameters of Oxidative Stress All selected allelochemicals, with the exception of coumarin, stimulated the total respiratory activity of primary roots (Table 3). This increase was due essentially to stimulation of the KCN-insensitive pathway. In comparison with the control, KCN-insensitive respiration increased nearly 4- to 5-fold in the presence of α-pinene, quercetin, and ferulic acid.

None of the assayed allelochemicals significantly affected the activity of SOD and APX (Fig. 4a and e). CAT activity

Table 1 Mean germination time, speed of germination, speed of accumulated germination of *Ipomoea triloba* L. incubated for 24, 48, or 72 hr in water (control), 1% (v/v) DMSO or in 500 μM allelochemicals

Allelochemical [500 μM]	Mean germination time ($\bar{t}$)	Speed of germination (S)	Speed of accumulated germination (AS)
	Seeds hours^{-1}		
Control	40.0±3.4	0.82±0.07	1.4±0.14
DMSO	34.7±2.0	0.79±0.07	1.3±0.03
Vanillic acid	32.6±2.6	0.74±0.01	1.3±0.02
ρ-Coumaric acid	37.5±2.9	0.71±0.04	1.2±0.06
Protocatechuic acid	48.0±4.9	0.75±0.11	1.2±0.14
Ferulic acid	39.6±3.9	0.68±0.06	1.2±0.10
Caffeic acid	33.6±1.6	0.78±0.03	1.4±0.04
Caffeine	41.3±2.3	0.76±0.03	1.3±0.06
Aconitic acid	37.1±2.8	0.72±0.05	1.3±0.09
Quercetin	38.2±1.1	0.73±0.06	1.3±0.12
Rutin	38.3±2.0	0.67±0.04	1.2±0.08
Coumarin	39.4±3.4	0.55±0.05	1.0±0.08
Eucalyptol	34.4±2.9	0.70±0.05	1.2±0.08
Camphor	38.6±2.2	0.69±0.06	1.2±0.08
α-Pinene	42.1±3.6	0.71±0.03	1.2±0.07
Limonene	34.7±1.6	0.81±0.03	1.4±0.03

Values are expressed as mean ± SE (N=5)

(Fig. 4b) was stimulated 42% by ferulic acid treatment. Alpha-pinene and ferulic acid treatment increased GR activity (Fig. 4d) by 45% and 54%, respectively. Guaicol peroxidase activity (Fig. 4c) was stimulated by treatment with α-pinene (+83%) and coumarin (+82%).

LOX activity was increased by treatment with α-pinene, quercetin, and ferulic acid (Fig. 4f). A stimulus of approximately 50% was observed in all treatments.

The data presented in Table 4 show that neither the content of glutathione nor the ratio between the reduced

Table 2 The effects of allelochemicals on root length, hypocotyl length, root fresh weight and hypocotyl fresh weight of *Ipomoea triloba L.* seeds were incubated for 48 hr, in the presence of water (control), 1% (v/v) DMSO or 500 μM allelochemicals

Allelochemical [500 μM]	Root length (cm)	Hypocotyl length (cm)	Root FW (mg per root)	Hypocotyl FW (mg per hypocotyls)
Control	1.43±0.01	1.16±0.14	8.92±0.12	12.24±1.53
DMSO	1.49±0.09	1.09±0.06	8.66±0.53	12.01±0.58
Vanillic acid	1.04±0.07*	1.08±0.08	7.74±0.21*	12.11±0.40
ρ-Coumaric acid	0.96±0.07*	1.31±0.09	7.39±0.35*	13.63±0.72
Protocatechuic acid	1.22±0.07	1.41±0.13	7.95±0.48	14.76±1.50
Ferulic acid	0.98±0.06*	1.09±0.06	6.31±0.17*	11.59±0.81
Caffeic acid	1.24±0.03	1.18±0.11	7.89±0.25	12.42±0.52
Caffeine	1.11±0.05*	1.29±0.06	7.83±0.22	13.59±0.95
Aconitic acid	1.26±0.04	1.20±0.10	8.73±0.19	12.55±0.75
Quercetin	1.12±0.08*	1.00±0.05	6.52±0.24*	11.07±0.54
Rutin	1.29±0.15	1.19±0.14	7.65±0.80*	12.53±1.70
Coumarin	0.57±0.08*	0.57±0.04*	7.48±0.79	9.870±1.02
Eucalyptol	1.26±0.08*	1.00±0.08	7.84±0.48	11.10±0.91
Camphor	1.10±0.05*	0.78±0.09*	7.69±0.09	9.355±1.13
α-Pinene	1.16±0.07*	1.01±0.05	6.81±0.34*	11.33±0.93
Limonene	1.24±0.07*	0.98±0.05	7.50±0.48	11.30±0.62

Values are expressed as mean ± SE (N=5). Significant differences between treated seeds and the respective controls are indicated. *$P \leq 0.05$, ANOVA with Duncan's multiple range test

Table 3 The effects of 500 μM α-pinene, coumarin, quercetin, or ferulic acid on the respiratory activity of primary roots of *Ipomoea triloba* L. Oxygen consumption in the absence (total respiration) or presence of 200 μM KCN (KCN-insensitive respiration). KCN-sensitive respiration: difference between total respiration and KCN-insensitive respiration

Conditions	Total respiration	KCN insensitive	KCN sensitive
	nmol $O_2 \times min^{-1} \times g$ fresh $weight^{-1}$		
Control	587.3±36.5	36.8±15.2	550.6±29.5
α-Pinene	964.2±105.6* (+64%)	187.3±11.2* (+408%)	775.9±104.3
Coumarin	694.0±30.2	153.3±20.1* (+316%)	540.7±30.6
Quercetin	913.4±105.4* (+56%)	156.7±18.9* (+325%)	762.4±107.6
Ferulic acid	907.7±103.5* (+55%)	187.9±25.0* (+410%)	719.7±94.6

The values are the means ± SE (N=6). Significant differences between means were identified by ANOVA with Duncan's multiple range test ($P \leq 0.05$). Symbols: (*) Values of the allelochemical-treated samples differing statistically from the control samples

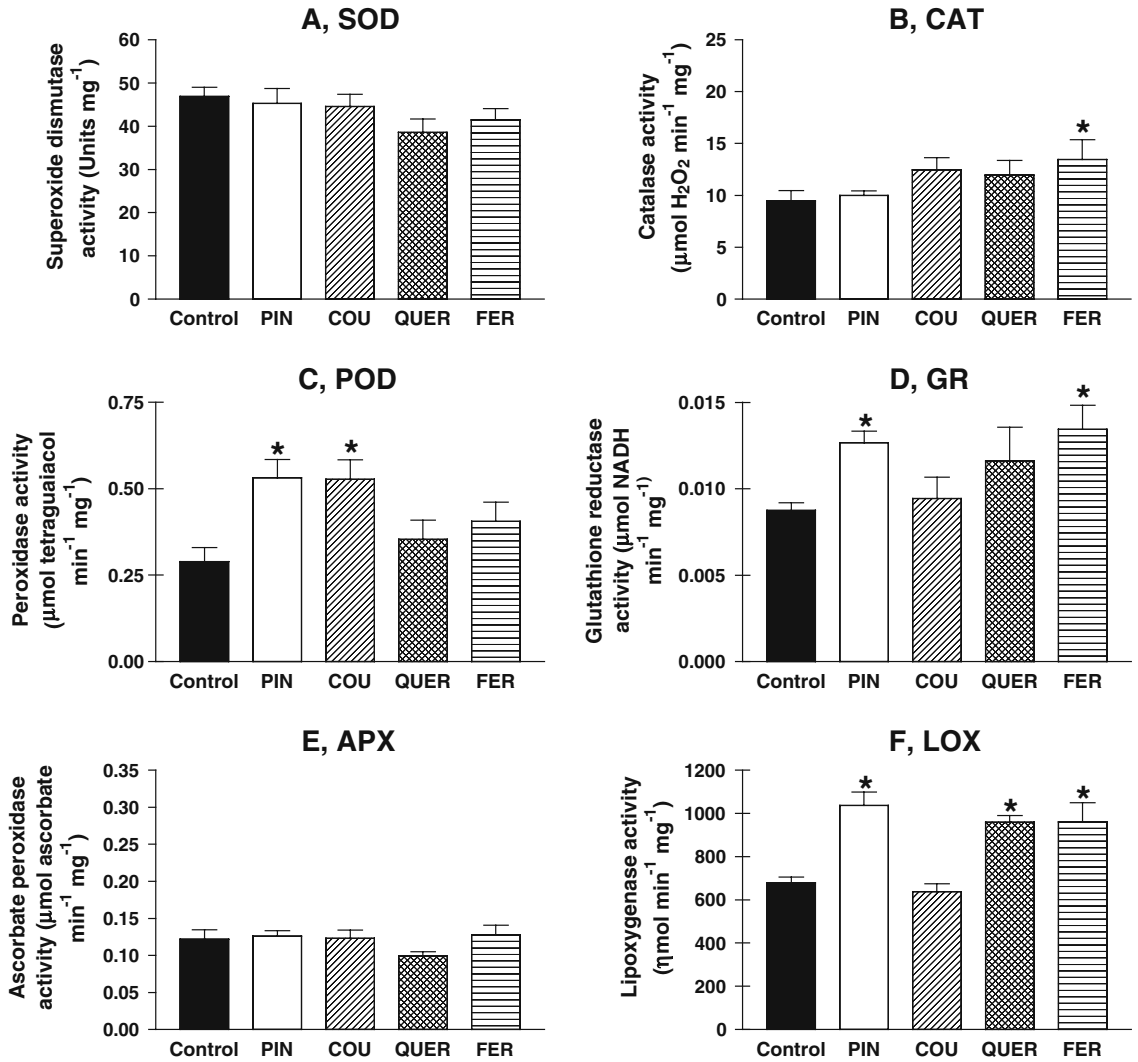


Fig. 4 Effects of 500 μM α-pinene, coumarin, quercetin or ferulic acid on activities of SOD (**a**), CAT (**b**), POD (**c**), GR (**d**), APX (**e**), and LOX (**f**) in roots of *Ipomoea triloba*. Seedlings were grown for 48 hr in the absence or presence of α-pinene, coumarin, quercetin, and ferulic acid (500 μM each). All values are the means ± SE (N=4–5). Significant differences between allelochemical-treated and untreated seedlings (Control) were identified by ANOVA with Duncan's multiple range test (*P<0.05)

Table 4 The effects of 500 μM α-pinene, coumarin, quercetin, or ferulic acid on the content of reduced and oxidized glutathione, GSH/GSSG ratio and malondialdehyde in roots of *Ipomoea triloba L.* seedlings grown for 48 hr

Allelochemical [500 μM]	Reduced glutathione (GSH) (nmol g root FW^{-1})	Oxidized glutathione (GSSG) (nmol g root FW^{-1})	GSH : GSSG ratio	MDA (nmol g root FW^{-1})
Control	121±20	265±20	461±10	5.2±0.2
α-Pinene	165±30	268±20	628±130	7.4±0.4* (+42%)
Coumarin	110±10	253±10	433±60	5.8±0.2
Quercetin	140±20	278±10	499±70	6.5±0.4
Ferulic acid	147±20	279±10	524±80	6.4±0.4

Values are expressed as mean ± SE (N=5). Significant differences between means were identified by ANOVA with Duncan's multiple range test ($P \leq 0.05$). Symbols: (*) Values of the allelochemical-treated samples differing statistically from the control samples

and oxidized forms were modified by any of the assayed allelochemicals. Alfa-pinene increased MDA content by 42%. Other allelochemicals did not change MDA content.

Discussion

Our results reveal that mitochondrial oxidative phosphorylation in embryos of *I. triloba* resumes shortly after the seed imbibition period, as indicated by considerable KCN-sensitive respiratory activity. After germination completion, the mitochondrial respiratory activity in cotyledons was increased further, probably due to increased metabolic activity linked to mobilization of reserved nutrients. The respiratory activity of primary roots, on the other hand, was similar to that of embryos, and it decreased gradually during the growth period. As the ADH activity in primary roots also decreased progressively, these findings most likely represent a decrease in ATP demand after germination completion. A similar pattern of changes in respiratory activity and ADH activity has been observed in two other weed species, *Bidens pilosa* L. (Pergo et al., 2008) and *Euphorbia heterophylla* L. (Kern et al., 2009).

The occurrence of SOD, CAT, and POD activities in embryos, along with significant KCN-insensitive respiration, suggests that production of reactive oxygen species is initiated as soon as mitochondrial respiration is resumed during seed imbibition. KCN-insensitive respiration accounts for oxygen that is converted into superoxide and H_2O_2 (Puntarullo et al., 1988), or utilized by mitochondrial alternative oxidase (AOX) and other cytosolic or membrane-bound oxidases, including lipoxygenases. Although the relative contribution of each process to KCN-insensitive respiration cannot be inferred from our data, all these processes have been demonstrated previously to be stimulated in oxidative stress conditions (Siedow and Moore, 1991; Baker and Orlandi, 1995; Thaler, 1999; Porta and Rocha-Sosa, 2002; Blokhin et al., 2003).

After the completion of germination, there was a distinct pattern of change in the activities of antioxidant enzymes in cotyledons and primary roots. The activity levels of SOD, CAT, GR, and POD in cotyledons remained similar to their levels in embryos. In primary roots, in contrast, the enzyme activities increased substantially over the experimental time period, possibly reflecting a further increase of ROS production. This probably was due to higher oxygen availability for primary roots in comparison with cotyledons, an interpretation that is in agreement with the observations of Wojtyla et al. (2006) in pea seeds. Those authors verified an increase in the concentration of free radicals in embryo axes after radicle protrusion, but they reported no significant changes in cotyledons. Goodman et al. (1986) also reported a large and rapid change in free radical levels in roots as a direct consequence of the oxygen content in the surrounding atmosphere.

The comparison of the activities of the assayed enzymes reveal that SOD and catalase are probably the main antioxidant enzymes involved in the scavenging of ROS in *I. triloba* during germination. The species also contains accessory enzymes that neutralize H_2O_2, such as the peroxidases. PODs exist in a large number of isoenzymatic forms, and they are implicated in a broad range of physiological processes (Passardi et al., 2005). Peroxidases bind to cell wall polymers by ionic or covalent interactions, and the bonds are believed to participate in lignin biosynthesis and the formation of cross-linking in the cell wall, (Müsel et al., 1997; Quiroga et al., 2000). Guaicol-specific PODs have been used as indicators of oxidative stress (Almagro et al., 2009).

It is unlikely that the ascorbate-glutathione cycle significantly contributes to H_2O_2 scavenging in *I. triloba* during seed germination. Glutathione reductase, which catalyses the last step of the cycle, presented low activity in the embryos and seedlings, and ascorbate peroxidase, the first enzyme in this cycle, appeared only in primary roots after germination completion (24 to 48 hr). There are other possible explanations for the presence of APX in *I. triloba*. It is known that ascorbate serves as a chemical scavenger for reactive oxygen in non-

enzymatic reactions (Noctor and Foyer, 1998), and a correlation between APX activity and the levels of ascorbic acid has been demonstrated by Wojtyla et al. (2006). Ascorbate, thus, could play a role in non-enzymatic antioxidant defense systems in primary roots of *I. triloba*.

LOX activity was not detected in embryos of *I. triloba*, but it appeared in cotyledons and primary roots after germination. LOX is another enzyme present in many isoforms in a wide range of organs and tissues, including in axes and cotyledons (Axelrod et al., 1981; Holtman et al., 1996; Feussner et al., 1997). The physiological roles of this family of enzymes in seed germination and seedling growth, however, are not completely known. It has been suggested that they function in tissue hydration (Hildebrand, 1989), cell elongation (Rodriguez-Conception et al., 1995), nitrogen storage (Tranbarger et al., 1991), and breakdown of storage lipids (Feussner et al., 1997). Because of the high lipid content that has been demonstrated in seeds of members of the *Ipomoea* genus (Stoller and Weber, 1970), the possibility exists that the higher activity of LOX in germinating seeds represents its role in lipid storage and mobilization. We cannot exclude, however, the possibility that LOX activity was increased in response to cellular oxidative stress. Lipid peroxidation has been reported to occur along with mitochondrial respiration during seed germination when the moisture content is higher than 0.25 g H_2O per g dry matter (Staniek and Nohl, 2000), and the activation of lipoxygenase is believed to be one of the immediate responses to changes in cell membrane structure induced by over production of reactive oxygen species (Siedow and Moore, 1991; Baker and Orlandi, 1995; Thaler, 1999; Porta and Rocha-Sosa, 2002; Blokhin et al., 2003).

In summary, the occurrence of SOD, CAT, POD, APX, and LOX in embryos and seedlings of *I. triloba* is probably related to ROS production resulting from mitochondrial metabolism resumption during germination and initial growth.

When the seeds were incubated in the presence of fourteen selected allelochemicals, each compound was observed to have different effects on *I. triloba*. At 500 μM, coumarin was the most active among all assayed compounds. The phenolic derivatives, vanillic acid, coumaric acid, ferulic acid, caffeine, and quercetin also inhibited primary roots growth, as did the terpenes eucalyptol, camphor, α-pinene, and limonene.

It should be mentioned that the range of activity of allelochemicals of different classes has been demonstrated to be variable (10^{-2} to 10^{-9}M), and differences of sensitivity among species also are common. Moreover, the allelochemical levels in the soil under natural conditions also can be considerably variable (Einhellig, 1995; Macías, 1995; Batish et al., 2006; Singh et al., 2006; Pergo et al., 2008). Nevertheless, the ecological relevance of an allelochemical activity at concentrations near 500 μM seems to be plausible (Einhellig, 1995; Macías, 1995).

The phytotoxic effect of α-pinene, quercetin, coumarin, and ferulic acid in the primary roots of *I. triloba* could be due, at least in part, to the induction of oxidative stress as indicated by the stimulation of antioxidant enzyme activities. α-Pinene increased the activities of GR and POD, coumarin stimulated POD activity, and ferulic acid stimulated the activities of GR and CAT. Also in agreement with this assumption, an increased lipoxygenase activity was found in the presence of α-pinene, quercetin, and ferulic acid.

The activation of H_2O_2 neutralizing enzymes is probably a defense mechanism that counters higher ROS production. Apparently, this antioxidant mechanism provides protection against oxidative stress induced by quercetin, coumarin, and ferulic acid, but not against oxidative stress induced by α-pinene because an increased malondialdehyde content was found only in the presence of this monoterpene. A higher production of ROS by 500 μM α-pinene might explain these different responses to assayed allelochemicals.

Differences in the mechanisms by which each allelochemical induced oxidative stress also should be considered. Mitochondria and chloroplasts are the main ROS sources (Puntarullo et al., 1988; Foyer et al., 1994; Mittler, 2002). It is possible that a direct action of α-pinene on mitochondria increases mitochondrial ROS generation, as this is in accordance with its effects found in the primary roots of maize (Abrahim et al., 2003a; Ishii-Iwamoto et al., 2006). Quercetin and ferulic acid also affect mitochondrial functions (Takahashi et al., 1998; Sert et al., 1998; Abrahim et al., 2003b). Although we have not measured the direct effects of these allelochemicals in mitochondria isolated from *I. triloba* seedlings, they both caused a strong stimulation of KCN-insensitive respiration of primary roots. Besides an increase in the fraction of oxygen converted to ROS, mitochondrial alternative oxidase (AOX) stimulation likely contributed to higher rates of KCN-insensitive respiration. AOX stimulation has been suggested as a compensatory mechanism that reduces ROS generation by the electron transport chain (Maxweel et al., 1999; Mittler, 2002; Clifton et al., 2005).

We emphasize that relatively little is known about the primary mechanism by which the allelochemicals induce oxidative stress in plant seedlings. Although molecular mechanisms have been reported (Bais et al., 2003; Weir et al., 2004; Golisz et al., 2008), it is difficult to discern whether they are causes or consequences of ROS production. For example, Bais et al. (2003) presented evidence that the allelochemical (-)-catechin triggers a wave of ROS that is initiated at the root meristem of *Arabidopsis thaliana*, leading to a Ca^{2+} signaling cascade followed by genome-wide changes in gene expression resulting in the death of the root systems. Golisz et al. (2008) also reported a higher expression of several genes including those that are up-regulated during biotic and/or abiotic stresses in *Arabidopsis thaliana* in

response to the allelochemicals fagomine, gallic acid, and rutin. Rudrappa et al. (2007) showed that gallic acid secreted by roots of the noxious weed *Phragmites australis* led to increased ROS that disrupted the root architecture of susceptible plants by affecting microtubule assembly. In *Chlorella vulgaris*, Qian et al. (2009) found that the oxidative stress caused by the allelochemical N-phenyl-2-naphthylamine is associated with photosynthesis inhibition and altered gene transcription.

Regardless of the primary mechanisms of allelochemical-induced oxidative stress, our data suggest that during seed germination and initial growth, a period when antioxidant enzyme activity increases and counteracts the harmful ROS effects produced during mitochondrial metabolism resumption, the presence of allelochemicals, which cause further oxidative stress, may leave the seeds/seedlings more vulnerable to cellular dysfunction and cell death.

In agro-ecosystems, allelochemicals with high oxidative stress-inducing potential can be released from cultivated plants or cover crops and cause inhibition of both growth and development of weed species. Identification of these allelochemicals may contribute to the discovery of natural compounds with herbicide potential (Duke et al., 2000; Inderjit and Duke, 2003). In fact, it is known that some commercial herbicides cause cellular death by inducing oxidative stress. This is a consequence of either electron flow interference in photosystem II (PSII) or the inhibition of anti-oxidant compound synthesis, including carotenoids (Kruse et al., 2006). It is reasonable to suggest that among the tested allelochemicals, α-pinene has a high potential to act as a herbicide in *I. triloba* through its effect in inducing oxidative stress during seed germination and the initial seedling growth.

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Hydrocarbons on Harvester Ant (*Pogonomyrmex barbatus*) Middens Guide Foragers to the Nest

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Abstract Colony-specific cuticular hydrocarbons are used by social insects in nestmate recognition. Here, we showed that hydrocarbons found on the mound of *Pogonomyrmex barbatus* nests facilitate the return of foragers to the nest. Colony-specific hydrocarbons, which ants use to distinguish nestmates from non-nestmates, are found on the midden pebbles placed on the nest mound. Midden hydrocarbons occur in a concentration gradient, growing stronger near the nest entrance, which is in the center of a 1–2 m diameter nest mound. Foraging behavior was disrupted when the gradient of hydrocarbons was altered experimentally. When midden material was diluted with artificial pebbles lacking the colony-specific hydrocarbons, the speed of returning foragers decreased significantly. The chemical environment of the nest mound contributes to the regulation of foraging behavior in harvester ants.

Key Words *Pogonomyrmex barbatus* · Hydrocarbons · Homing · Chemical ecology · Foraging behavior

Introduction

Ant foragers use both individual and collective processes to find their way to and from the nest. The best-known example of navigation by foraging ants uses both. First, a scout navigating on its own searches for food, and when it finds food, returns to the nest while laying a chemical trail. The trail initiates a collective process that guides many other ants to the food source, while each ant lays a trail on the way back. In some species, this collective process regulates the number of foragers according to the abundance of food (Walsh et al., 1965). Other species use a different collective process that involves chemical trails. For example, workers of the polydomous Argentine ant *Linepithema humile* lay trail pheromone as they travel (Aron et al., 1989), thus leading all the ants to follow a shared trail system from one nest to another.

In the desert seed-eating ant, *Pogonomyrmex barbatus*, foragers do not use trail pheromone to recruit to specific locations (Greene and Gordon, 2007). Instead, foragers leave the nest together in a direction influenced by the patrollers, a small group of ants that scout the foraging area early in the morning, and a forager's memory of where it last collected food. The patrollers place a chemical cue from the Dufour's gland on about 20 cm of the nest mound, and this influences the directions foragers will travel up to 20 m from the nest (Greene and Gordon, 2007). The choice of direction allows colonies to avoid overlap with the foragers of neighboring conspecific colonies (Gordon, 1992; Gordon and Kulig, 1996). Each forager leaves the nest with a stream of other ants and searches for food. Once it finds food, it returns immediately to the nest. The colony uses another process, the rate of forager return, to regulate foraging activity; foragers are stimulated to leave the nest by the return of other foragers with food (Schafer et al., 2006; Gordon et al., 2008). The rate of forager return is a measure of the availability of food, because the more food is available, the more quickly foragers find it and return to the nest. Since foraging is regulated by the rate of return of

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successful foragers, any process that facilitates the rapid return of foragers would promote the accuracy of this measure of food availability.

Olfactory cues on the nest mound or foraging area of ants play an important role in orientation and homing behavior (Cammaerts and Cammaerts, 1987; Wu, 1989; Jaffé et al., 1990). Cuticular hydrocarbons are vital chemical cues used by ants in discrimination between nestmates and non-nestmates (Singer, 1998; Wagner et al., 2000; d'Ettorre and Lenoir, 2010). Hydrocarbons (HCs) have been found in and on the nests of some ant species (Grasso et al., 2005; Lenoir et al., 2009). Hydrocarbons are transferrable, stable, species- and colony-specific compounds that make them ideal candidates for homing signals on the nest mounds of ants.

Nests of *P. barbatus* have large, flat, circular mounds about 1–2 m in diameter (Fig. 1a). Harvester ant nest mounds are generally, but not always, covered with small midden pebbles collected by the foragers (Fig. 1b; see Gordon, 1984a). The midden pebbles surround the nest entrance, which is located approximately in the center of the nest mound. Mounds with midden pebbles typically are cleared of vegetation by the ants.

Observations suggest that midden material is coated with HCs. Harvester ants of a related species, *Pogonomyrmex badius,* collect bits of charcoal, which absorb organic compounds, for their nest middens. The charcoal middens prevent incursion on the mound by ants of other species (Gordon, 1984b), which suggests that midden material may contain species-specific HCs or other discriminatory olfactory cues. Hölldobler, (1971) found that secretions from the Dufour's gland act as homing signals for *P. badius* foragers locating the nest. Dufour's gland secretions in several *Messor* harvester ant species contain HCs (Brand and Mpuru, 1993; Co et al., 2003; Tullio et al., 2003).

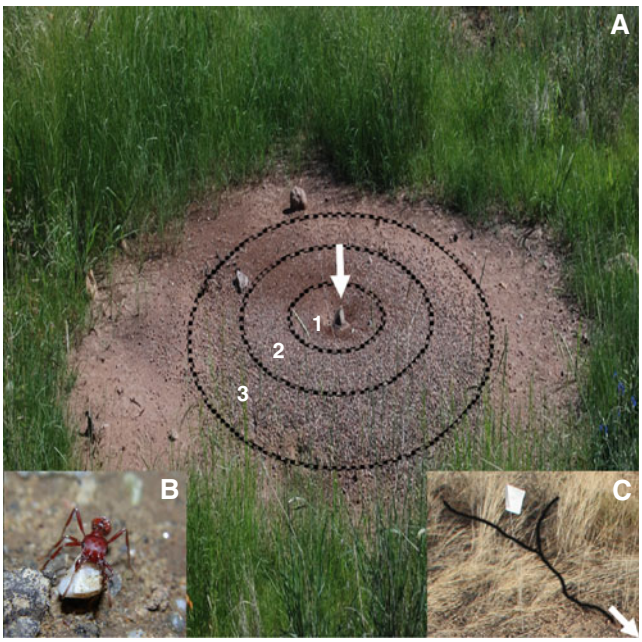


Fig. 1 **a** Anatomy of a *Pogonomyrmex barbatus* nest mound. Midden zones are indicated by dashed circles; 1 = center, 2 = middle, and 3 = outer zone. The white arrows indicate the nest entrance (center of midden zone 1). **b** Photograph in the lower left corner shows a *P. barbatus* forager carrying a midden pebble. **c** Photograph located in lower right corner shows an established foraging trail at the edge of the nest mound. The black line marks the trail. The white arrow points towards the nest entrance

Midden workers of *P. barbatus* arrange midden pebbles on the nest mound (Gordon, 1984a), and have been observed manipulating midden pebbles with their mouthparts (Gordon and Mehdiabadi, 1999). Hydrocarbons can be passively distributed through contact with the ant cuticle (Soroker et al., 1995; Vienne et al., 1995) or secreted onto objects from glands inside the ant (Soroker et al., 1995, 2003). It is unclear whether ants are actively or passively marking midden pebbles. Returning foragers can be seen attennating midden pebbles on their way back to the nest entrance (SJS personal observation), which suggests that there may be a spatial pattern in the concentration of midden HCs.

Here, we examined how HCs on *P. barbatus* nest mounds influence the speed at which foragers travel from the edge of the nest mound to the nest entrance. (1) We tested for the presence of HCs in *P. barbatus* midden pebbles. (2) We tested whether ants can discriminate between their own midden HCs and foreign midden HCs. (3) We examined the spatial pattern of HC abundance in midden pebbles on the nest mound. (4) To determine the effects of nest mound chemical cues on foraging behavior, we experimentally manipulated the distribution of midden material.

Methods and Materials

Study Site. All experiments were conducted with *P. barbatus* colonies at the site of a long-term field study near Rodeo, NM, USA, in which colonies have been censused since 1985 so that colony ages are known (census methods in Gordon and Kulig, 1996).

1 Are there Hydrocarbons (HCs) in the Midden?

Hydrocarbon Extraction and Analysis To determine whether midden pebbles contain HCs, midden pebbles were collected from 10 mature *P. barbatus* colonies (ages 6–18 year). HCs were extracted from 5 g of midden pebbles by immersing the pebbles in 5 ml of 100% pentane (Sigma, St. Louis, MO, USA) for 30 min. For comparison, ant cuticular HCs were extracted from individual ants by immersing the ants in 0.5 ml of 100% pentane (Sigma) for 7 min. Suspensions of

both midden pebbles and ants were lightly shaken during the first and last minute of the soak period. The suspension solution was eluted from a 1-cm silica gel (28–200 mesh, Sigma) column with a 1 ml wash of 100% pentane. The eluant was dried overnight, and the samples were redissolved in 100 µl of pentane for midden pebble HCs or 25 µl of pentane for ant cuticular HCs, including an internal standard (27 ng per µl of triatriacontane,$C_{33}H_{68}$; Fluka Analytical, Buchs, Switzerland). The amounts of midden HCs were quantified by comparing their peak areas with that of the internal standard. Samples were analyzed by gas chromatography (GC). GC analyses were performed on a Varian 3900 GC with a CP-8400/8410 auto sampler and a flame ionization detector (Tissot et al., 2001; Greene and Gordon, 2003). Aliquots of 8 µl were introduced by split-less injection onto a capillary column (DB-1, 30 m, 0.25 mm ID, 0.25-µm film thickness; Agilent J&W Scientific, Santa Clara, CA, USA), with helium as the carrier gas flowing at 1 ml/min. The injector temperature was 300°C. The temperature was 170°C during injection, and was then increased to 220°C at 25°C/min, 220°C–310°C at 3°C/min. Retention times were verified on the GC for 6 *n*-alkanes using a standard mixture containing *n*-tricosane, *n*-tetracosane, *n*-pentacosane, *n*-heptacosane, *n*-hexacosane, and *n*-nonacosane (Sigma) interspersed among runs.

There were no readily available standards for methyl alkane and alkene identification. Tentative methyl alkane and alkene identifications were based on analyses of a subset of midden pebble (N=3), and *P. barbatus* worker (N=3) HC samples by coupled gas chromatography/mass spectrometry (GC/MS), and by cross reference with previously published work (Wagner et al., 1998; Tissot et al., 2001). GC/MS was conducted at the Department of Environmental Sciences, Policy and Management at the University of California Berkeley, using a 7890A Agilent GC (Agilent Technologies, Santa Clara, CA, USA) coupled with a 5975 C Agilent Mass Selective Detector (electron impact ionization at 70 eV). Aliquots of 1 µl were injected by using an Agilent 7683B automatic injector operated in split mode (5:1) onto a capillary column (DB-5, 30 m, 0.32 mm ID, 0.25-µm film thickness; Agilent J&W Scientific), with helium as the carrier gas flowing at 0.7 ml/min. Mass Selective Detector (MSD) scanning parameters ranged from 50 to 550 amu, with a sampling rate of 2.91 scans/sec and a threshold detection of 150 counts.

Statistical Analysis Nest mounds were divided into three zones; center, middle, and outer, for all colonies (Fig. 1a; 3 zones per colony, 10 colonies, N=10 replicates per midden zone). The average concentrations in nanograms (SD) of each HC and HC class present in the midden pebble samples were calculated for each midden zone. Nonparametric Kruskal Wallis one-way ANOVAs were used to compare the average individual and average total HC concentrations of the 3 midden zones. To compare the concentrations of HCs on midden pebbles and on forager cuticles, the average concentration in ng (SD) of each HC and HC class was calculated for 50 *P. barbatus* foragers.

2 Can *P. barbatus* Workers Discriminate between their own Midden and that of Another Nest?

Bioassay We tested whether harvester ants recognize chemical cues on midden pebbles, and HCs extracted from the surface of midden pebbles, by measuring the ants' agonistic response to midden pebbles and objects coated with a chemical stimulus (Wagner et al., 2000). Stimulus objects, either a pile of midden pebbles or a 5×5×10 mm glass block (Wagner et al., 2000) coated with a chemical stimulus, were placed on the nest mound approximately 10 cm from the nest entrance, but never directly along a foraging trail. After placement of the stimulus objects, we recorded every 30 sec for 5 min the number of ants biting or stinging the objects, and the total number of ants in contact with the objects, either with antennae, abdomen, legs, or any other body part.

Trials were conducted on 4–6 August 2001 and 8–17 May 2002. We measured the proportion of ants that exhibited agonistic behavior, biting, and stinging. The first experiment tested the response of workers to midden pebbles of their own and of another nest. About 20 midden pebbles were placed in a small pile on the nest mound. On a single day, colonies (ages 3–16 year; N=15 colonies) were offered midden pebbles from its own nest, and from another nest, once in a randomized sequence. The second experiment tested the response of workers to midden HCs and worker cuticular HCs of their own and of another colony. On a single day, colonies (ages 3–16 year; N=8 colonies) were offered one 5×5×10 mm glass block either coated with nestmate cuticular HCs from same-colony workers, non-nestmate cuticular HCs from foreign-colony workers, HCs from same-colony midden pebbles, HCs from foreign-colony midden pebbles, and pentane (solvent control), once in a randomized sequence.

Statistical Analysis For both experiments, we calculated the angular transformation (Zar, 1999) of the proportion of ants that displayed either biting or stinging, out of the total number of ants that made contact with the pile of midden pebbles or the glass blocks coated with a chemical stimulus. For the first experiment, the angular transformed data were compared for the two midden pebble treatments (same-colony and foreign-colony midden pebbles), using a *t*-test. For the second experiment, all 5 treatments (same-colony worker cuticular HCs, foreign-colony worker cuticular

HCs, same-colony midden pebble HCs, foreign-colony midden pebble HCs, and solvent control) were compared using an ANOVA with Fisher's PLSD for *post hoc* analysis.

3 Is there a Spatial Pattern of Hydrocarbon Abundance in Midden?

Midden Collection Midden pebbles were collected from 10 mature *P. barbatus* colonies (ages 6–18 year) in August 2008. Pebble cover on mounds, estimated by sight, ranged from 40–100% of the total mound surface area, with the remainder of the nest mound as bare soil. Nest mounds were defined as the entire area around the nest entrance of a colony extending either to the edge of the vegetation or of the pebble cover. Nest mounds were divided into 3 zones of approximately similar radii: center (closest to nest entrance), middle, and outer (Fig. 1a). From each zone, we gathered ~10 g of midden pebbles using nylon gloves, and placed the midden pebbles into separate plastic bags. Analysis of samples stored in the bags indicated that there was no detectable contamination from the plastic. In each of the three zones, on five nest mounds, ~2 g of soil were collected from the surface beneath the midden pebbles. Samples were stored at −20°C. All samples were transferred to 16×100 mm glass culture tubes (VWR Scientific Products, West Chester, PA, USA) before HC extraction. Five grams of midden pebbles and 2 g of surface soil were extracted by immersing the pebbles or soil in 5 ml of 100% pentane (Sigma) for 30 min. Hydrocarbon extraction and analysis of both midden pebbles and surface soil were performed as above. Samples were redissolved in 100 μl of pentane before GC analysis.

Statistical Analysis We calculated the mean total HC concentration in ng (SD) for midden pebbles and surface soil from each zone. Nonparametric Kruskal Wallis one-way ANOVAS were used to test for differences between midden zones. Dunn's Multiple Comparison tests were used for post-hoc analysis.

4 Does the Hydrocarbon Gradient in the Harvester Ant Midden Influence Foraging Behavior?

Colony Selection Twenty colonies, all at least 5 year old, were selected in August 2009. Nest mound size ranged from 2.62 m^2 to 10.50 m^2, and pebble cover ranged from 50–99%. Each colony was located at least 10 m away from the nearest neighboring colony. All nest mounds were approximately circular. The area of each nest mound was calculated using the longest chord of the irregularly-shaped circular nest mounds.

Data Collection before Experimental Manipulation We measured the time foragers took to reach the nest entrance from the edge of the mound. In all 20 colonies, measurements were made during peak foraging hours, from 7 am to 10:45 am daily, for 5 day from 12–16 August 2009. Because foraging activity changes in the course of the activity period (Gordon, 2002), the sequence of colonies in which foraging was observed was reversed from 1 day to the next. For each colony we chose a foraging trail cleared of vegetation that had been used by foragers for at least two consecutive days (Fig. 1c). A flag was placed at the perimeter of the nest mound to mark the beginning of the trail. Distances from the flag to the nest entrance ranged from 0.84 m to 2.03 m. We recorded the time it took individual foragers to return to the nest entrance from the flag, and calculated forager speed as the return time divided by the distance from the flag to the nest entrance. We counted only returning foragers, which could be distinguished from other workers because the returning forager carried a food item in its mandibles. For each colony, and each of the 5 day of observation before treatment, we measured the speeds of 3 foragers. In a total of 60 observations from 10 of the 20 colonies, there was no foraging activity, and these 60 observations were excluded. A total of 240 observations from 20 colonies were used in the analysis.

Experimental Manipulations To determine the effect of chemical cues on the return of foragers, we either (a) diluted nest middens by adding an inert substance to the nest mound, or (b) deprived nests of midden material. To test the effect of colony-specific HCs on forager return speed, we diluted midden pebbles with expanded Perlite (Scotts Miracle-Grow®, Maryville, OH, USA), a substance similar in structure and size but lacking colony-specific HCs. Perlite is an amorphous volcanic glass that has been softened under extreme heat for commercial use. Perlite consists of 70–75% silicon dioxide, 12–15% aluminum oxide, 3–4% sodium oxide, 3–5% potassium oxide, and trace amounts of iron oxide, magnesium oxide, and calcium oxide. Chemically, there are only minor differences between Perlite and midden pebbles. Midden pebbles found on *P. barbatus* nest mounds and Perlite are both volcanic rocks which contain the same major elements found in all silicate minerals (Elston et al., 1973). Expanded Perlite clumps together to form small balls that are roughly the size of midden pebbles. To deprive nests of midden material, we removed all midden pebbles from the nest mound and left the soil bare.

Nest mounds were divided into 3 zones of approximately similar radii: center (closest to nest entrance), middle, and outer (Fig. 1a). There were 4 treatments: 1) Midden pebbles in the center and middle zones were diluted with Perlite. The center and middle zones were combined because the

results of the experiment described in the previous section showed no significant difference between the zones in HC concentration. 2) Midden pebbles in the outer zone were diluted with Perlite. 3) Midden pebbles for all 3 zones were diluted with Perlite. In treatments 1, 2, and 3, we applied Perlite to the indicated zones twice, on days 1 and 4 of the 6-d observation period. Perlite was reapplied after 2 day because in all colonies, the Perlite treatment was almost completely removed by d 3, probably due to wind, rain, and the activity of nest maintenance workers in the afternoon, when no observations of foraging activity were made. 4) All midden pebbles were removed, leaving the bare soil that had been covered by midden pebbles.

An alternative design would have been to remove pebbles, extract HCs and return them to mounds. This was not feasible due to the large quantity of pebbles on the mound and because extracted pebbles would have been difficult to return to the mound in a homogenous manner.

Data Collection after Experimental Manipulation We measured the speed at which foragers returned to the nest entrance from the edge of the mound using the methods described above once a day for 6 day from 17–22 August 2009. In a total of 76 observations from 11 of the 20 colonies, the colony was not foraging actively, and these observations were excluded. A total of 284 observations of forager return speeds from the 20 colonies were used in the analysis.

Statistical Analysis For each treatment, we compared forager return speeds before and after treatment within each treatment group using unpaired *t*-tests. Data were square-root transformed to meet the assumptions of normality.

To compare forager return speeds in the 4 treatment groups, we first normalized for variation among colonies in forager speed. We found for each colony the difference between mean forager return speed before and after treatment, and divided this difference by that colony's mean forager return speed. Using the normalized mean colony difference in forager return speed before and after treatment, we tested for differences among treatments using an ANOVA.

Results

1 Are there Hydrocarbons (HCs) in the Midden?

Midden pebbles contained HCs tentatively identified as identical to those found on the ants' cuticles (Fig. 2). Twenty-one HCs that occur on the *P. barbatus* cuticle were detected in the midden (Table 1). It is possible that other HCs were also present in trace amounts. Hydrocarbon compounds were of four classes, *n*-alkanes, monomethylalkanes, dimethylalkanes, and alkenes. HCs present in the highest abundances were *n*-alkanes. The 5 most abundant HCs in the midden were *n*-hentriacontane (C31), *n*-nonacosane (C29:0), *n*-pentacosane (C25:0), *n*-heptacosane (C27:0), and *15-, 13-, 11-, 9*-methylhentriacontanes (C31:0), respectively. There was an overall difference among midden zones in individual HC concentration for all HCs except *n*-tricosane and *n*-tetracosane. There was a significant difference among midden zones in the average concentration of HCs for each HC class (Kruskal Wallis tests: alkanes: H_3=8.87, N=10, P=0.012; monomethylalkanes: H_3=11.66, N=10, P=0.003; dimethylalkanes: H_3=10.55, N=10, P=0.005; alkenes: H_3=12.21, N=10, P=0.002). There were about 10 times more HCs on one forager's cuticle than on 5 g of midden pebbles; the mean concentration of HCs in ng (SD) on one forager's cuticle, and on 5 g of midden pebbles were 562.30 (252.27) and 57.81 (67.78), respectively.

2 Can *P. barbatus* Workers Discriminate between their own Midden and that of Another Nest?

Midden pebbles contain colony-specific HCs that are recognized by workers. Ants were more likely to respond aggressively toward midden pebbles from another colony's nest than toward midden pebbles from their own colony's nest (Fig. 3a; Paired *t*-test: t_{26}=4.1, P<0.001). The mean proportion of ants biting/stinging (SD) midden pebbles from their own and conspecific nests were 0.06 (±0.08) and 0.42 (±0.19), respectively.

Pogonomyrmex barbatus workers recognized the HC blend extracted from midden pebbles of their own colony. Workers distinguished HCs extracted from midden pebbles of their own nest from those extracted from the midden pebbles, or workers of another colony. A higher proportion of contacts were aggressive in response to extracts of midden pebbles and whole ants from other colonies than to extracts of midden pebbles or extracts from ants of their own colony (Fig. 3b; ANOVA: $F_{4,\ 35}$=8.8, P<0.001). The mean proportions of workers biting/stinging (SD) glass blocks treated with nestmate cuticular HCs from same-colony workers, non-nestmate cuticular HCs from foreign-colony workers, HCs from same-colony midden pebbles, HCs from foreign-colony midden pebbles, and solvent were 0.40 (±0.33), 0.84 (±0.18), 0.40 (±0.23), 0.84 (±0.27), and 0.24 (±0.20), respectively [Fisher's PLSD (Protected Least Signficant Difference): non-nestmate (foreign) cuticular HCs (FCHCs), foreign midden HCs (FMHCs): mean difference = 11.606, P=0.262; cuticular HCs from nestmates (CHCs), colony midden HCs (MHCs): mean difference = 17.249, P=0.099; CHCs, solvent: mean difference = 11.953, P=0.249; MHCs, solvent (pentane): mean difference = 5.296, P=0.606; FCHCs vs CHCs, MHCs, solvent: mean difference = 32.511, 49.760, and 44.464, P=0.003, <0.001, and <0.001 respectively;

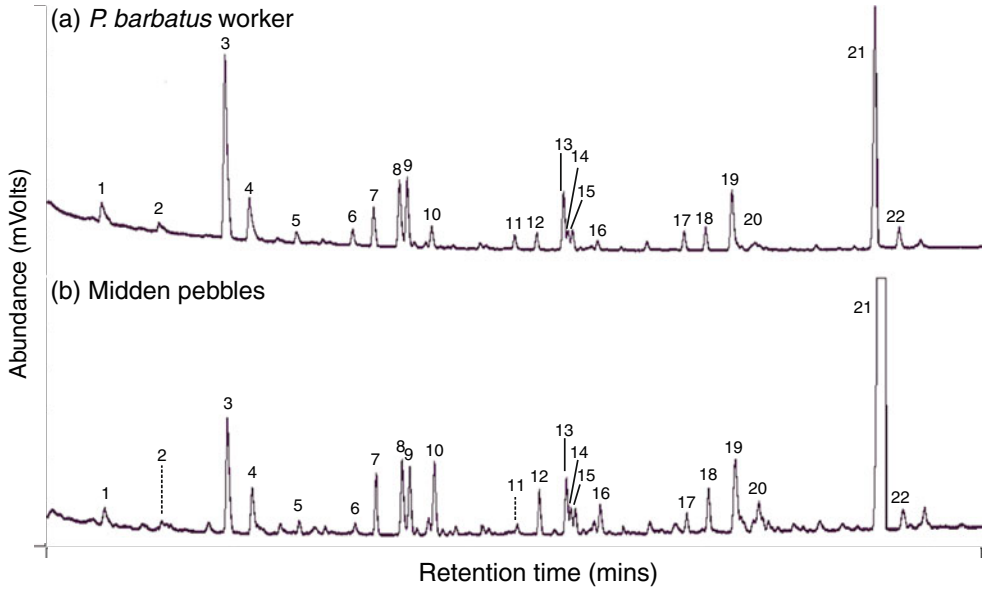


Fig. 2 Gas chromatograms of *Pogonomyrmex barbatus* forager (**a**), and of midden pebbles from a *P. barbatus* nest mound (**b**). Each number identifies a hydrocarbon peak: **1**– *n*-tricosane, **2**– *n*-tetracosane, **3**– *n*-pentacosane, **4**– 13-methylpentacosane*, **5**– *n*-hexacosane, **6**– heptacosene*, **7**– *n*-heptacosane, **8**– 13-methylheptacosane*, **9**– 7-methylheptacosane*, **10**– 7,23-dimethylheptacosane*, **11**– nonacosene*, **12**– *n*-nonacosane, **13**– 15-methylnonacosane*, **14**– 9-methylnonacosane*, **15**– 7-methylnonacosane*, **16**– 7,13-dimethylnonacosane*, **17**– hentriacontene*, **18**– *n*-hentriacontane*, **19**– 15-,13-,11-,9-methylhentriacontanes*, **20**– 13,17-, 11,15-, 9,13-dimethylhentriacontanes*, **21**– *n*-triatriacontane (internal standard), **22**– 17-,15-,13-,11-,9-,7-methyltritriacontanes*. *Compounds tentatively identified based on mass spectral interpretation and prior literature

FMHCs vs CHCs, MHCs, solvent: mean difference = 20.905, 38.154, and 32.858, P=0.047, <0.001, and 0.003, respectively].

3 Is there a Spatial Pattern of Hydrocarbon Abundance in Midden?

There is a HC concentration gradient on the midden pebbles and on the soil below the midden pebbles on the surface of nest mounds. Hydrocarbon concentration in both the midden pebbles and soil is higher near the nest entrance (Fig. 4). The mean total HCs in ng (SD) for the midden pebbles were 82.75 (56.00), 73.73 (92.97), and 16.94 (14.03) for the center, middle, and outer zones, respectively (Kruskal Wallis test, H_3=10.41, N=10, P=0.006); the center zone differed significantly from the outer zone (Dunn Multiple Comparison test, difference in rank sum = 12.2, P<0.01). The mean total HCs in ng (SD) for the soil were 40.08 (13.84), 11.91 (6.15), and 8.20 (2.14) for the center, middle, and outer zones, respectively (Kruskal Wallis test: H_3=9.78, N=5, P=0.008); the center and outer zone differed significantly (Dunn Multiple Comparison test, difference in rank sum = 8.6, P<0.01).

4 Does the Hydrocarbon Gradient in the Harvester Ant Midden Influence Foraging Behavior?

The HCs on the nest mound facilitate the return of foragers to the nest. Diluting midden pebbles with Perlite reduced the speed with which returning foragers reached the nest entrance (Fig. 5; Unpaired t-test: t_{522}= 3.689, P<0.001). The mean speed at which returning foragers reached the nest entrance (SD) before and after treatment was 5.9 (3.0) cm/s (N=240) and 5.0 (3.0) cm/s (N=284), respectively. The average forager speed to nest entrance (SD) in the center and middle zones (treatment 1) was 6.4 (3.3) cm/s before treatment and 5.1 (3.1) cm/s after treatment (*Unpaired t-test:* t_{132}=2.21, P=0.029). In the outer zone (treatment 2), the average speed to the nest entrance (SD) before treatment was 5.4 (2.3) cm/s and 4.0 (2.0) cm/s after treatment (Unpaired t-test: t_{136}=3.99, P=0.001). In all 3 zones (treatment 3), the average speed to the nest entrance (SD) before treatment was 6.6 (3.5) cm/s and 5.1 (3.0) cm/s after treatment (Unpaired t-test: t_{124}=2.589, P=0.011). The speed of returning foragers to the nest entrance increased slightly but not significantly after the removal of pebbles, with bare soil remaining (treatment 4). When all pebbles were removed, the average speed of returning foragers to the nest entrance (SD) before treatment was 5.1 (2.7) cm/s and 5.8 (3.2) cm/s after treatment (Unpaired t-test: t_{124}=1.17, P=0.243).

Forager return speed did not differ according to the midden zone from which Perlite was added. Diluting the midden with Perlite (treatments 1–3) reduced forager return speeds more than the removal treatment (treatment

Table 1 The mean concentration (ng) of all hydrocarbons present in each midden zone (center, middle, and outer) and on the cuticle of *Pogonomyrmex barbatus* foragers

Hydrocarbons	Formula	Hydrocarbon concentration (ng)[a]					
Alkanes	C_nH_{2n+2}	Center	Middle	Outer	p-value[b]	All Zones[c]	*P. barbatus* forager[d]
1. *n*-Tricosane	$C_{23}H_{48}$	**3.46** (3.15)	**2.53** (2.08)	**1.96** (1.42)	*P*=0.31	**2.65** (2.33)	**31.37** (15.15)
2. *n*-Tetracosane	$C_{24}H_{50}$	**1.22** (0.91)	**1.10** (1.20)	**0.51** (0.43)	*P*=0.07	**0.94** (0.93)	**12.42** (6.43)
3. *n*-Pentacosane	$C_{25}H_{52}$	**13.56** (10.32)	**7.56** (8.48)	**2.95** (1.39)	*P*=0.005**	**8.02** (8.69)	**208.26** (105.76)
5. *n*-Hexacosane	$C_{26}H_{54}$	**1.91** (0.99)	**1.04** (0.99)	**0.64** (0.59)	*P*=0.008**	**1.20** (1.00)	**11.28** (5.49)
7. *n*-Heptacosane	$C_{27}H_{56}$	**6.98** (4.35)	**6.34** (5.65)	**1.98** (1.35)	*P*=0.02*	**5.10** (4.63)	**37.29** (20.52)
12. *n*-Nonacosane	$C_{29}H_{60}$	**5.92** (4.45)	**17.37** (32.60)	**2.03** (2.46)	*P*=0.04*	**8.44** (19.54)	**13.63** (7.07)
18. *n*-Hentriacontane*	$C_{31}H_{62}$	**6.01** (5.09)	**20.66** (33.84)	**2.88** (5.14)	*P*=0.02*	**9.85** (20.82)	**14.24** (8.21)
Mean alkanes	C_nH_{2n+2}	**39.06** (23.43)	**56.60** (76.87)	12.96 (10.02)	*P*=0.012*	**155.17** (110.04)	**331.83** (163.53)
Total alkanes	C_nH_{2n+2}	356.42	566.00	129.59		1086.15	16424.45
Methyl alkanes	$C_{n+1}H_{2n+5}$						
4. 13-Methylpentacosane*	$C_{26}H_{55}$	**4.25** (3.72)	**1.54** (1.86)	**0.66** (0.61)	*P*=0.005**	**2.03** (2.66)	**30.37** (16.66)
8. 13-Methylheptacosane*	$C_{28}H_{59}$	**5.83** (5.27)	**2.11** (3.02)	**0.70** (0.79)	*P*=0.01**	**2.72** (3.87)	**33.71** (16.08)
9. 7-Methylheptacosane*	$C_{28}H_{59}$	**5.17** (4.57)	**1.71** (2.55)	**0.63** (0.95)	*P*=0.009**	**2.36** (3.38)	**34.00** (16.26)
13. 15-Methylnonacosane*	$C_{30}H_{63}$	**4.05** (3.50)	**1.38** (2.17)	**0.31** (0.52)	*P*=0.006**	**1.82** (2.74)	**26.61** (13.11)
14. 9-Methylnonacosane*	$C_{30}H_{63}$	**1.62** (1.53)	**0.96** (1.52)	**0.10** (0.20)	*P*=0.03*	**0.85** (1.33)	**8.87** (4.21)
15. 7-Methylnonacosane*	$C_{30}H_{63}$	**1.75** (1.44)	**0.57** (0.91)	**0.11** (0.24)	*P*=0.006**	**0.76** (1.13)	**9.11** (4.77)
19. 15-,13-,11-,9-Methylhentriacontanes*	$C_{32}H_{67}$	**6.61** (5.55)	**2.52** (3.09)	**0.57** (0.72)	*P*=0.003**	**3.11** (4.32)	**35.15** (21.59)
21. 17-,15-,13-,11-,9-,7-Methyltritriacontanes*	$C_{34}H_{71}$	**1.62** (1.28)	**0.94** (1.04)	**0.06** (0.19)	*P*=0.004**	**0.85** (1.12)	**10.70** (6.81)
Mean methyl alkanes	$C_{n+1}H_{2n+5}$	**30.89** (26.24)	**11.73** (14.83)	**3.13** (3.96)	*P*=0.003**	**57.19** (28.71)	**191.20** (94.77)
Total methyl alkanes	$C_{n+1}H_{2n+5}$	287.07	117.33	31.32		457.54	9426.45
Dimethyl alkanes	$C_{n+2}H_{2n+8}$						
10. 7,23-Dimethylheptacosane*	$C_{29}H_{62}$	**5.54** (4.54)	**2.12** (2.62)	**0.69** (0.66)	*P*=0.006**	**2.64** (3.42)	**13.32** (16.32)
16. 7,13-Dimethylnonacosane*	$C_{31}H_{66}$	**2.04** (1.86)	**0.77** (1.13)	**0.10** (0.22)	*P*=0.008*	**0.92** (1.41)	**3.71** (2.21)
20. 13,17-, 11,15-, 9,13-Dimethylhentriacontanes*	$C_{32}H_{67}$	**1.84** (1.98)	**0.55** (0.92)	**0.06** (0.20)	*P*=0.012*	**0.79** (1.43)	**4.52** (4.98)
Mean dimethyl alkanes	$C_{n+2}H_{2n+8}$	**9.42** (8.26)	**3.44** (4.58)	**0.85** (0.97)	*P*=0.005**	**45.71** (32.80)	**21.55** (20.54)
Total dimethyl alkanes	$C_{n+2}H_{2n+8}$	87.54	34.38	8.52		137.13	1077.27
Alkenes	C_nH_{2n}						
6. Heptacosene*	$C_{27}H_{54}$	**1.05** (0.71)	**0.33** (0.73)	**0.00** (0.00)	*P*<0.001***	**0.44** (0.69)	**7.24** (4.68)
11. Nonacosene*	$C_{29}H_{58}$	**0.69** (0.59)	**0.81** (1.11)	**0.00** (0.00)	*P*<0.001***	**0.48** (0.78)	**6.48** (3.81)
17. Hentriacontene*	$C_{31}H_{62}$	**1.64** (1.28)	**0.81** (1.12)	**0.00** (0.00)	*P*=0.002**	**0.77** (1.10)	**10.02** (5.74)
Mean alkenes	C_nH_{2n}	**3.38** (2.51)	**1.95** (2.80)	**0.00** (0.00)	*P*=0.002**	**17.78** (5.88)	**23.74** (13.60)
Total alkenes	C_nH_{2n}	31.05	19.53	0.00		53.35	1186.80

[a] Values in bold represent the mean hydrocarbon concentration (per 5 g midden pebbles or ant) and values in parentheses represent the standard deviation

[b] *P*-values were obtained by comparing the mean hydrocarbon concentrations among all midden zones using one-way non-parametric Kruskal Wallis ANOVAs

[c] Values represent the mean concentration of each hydrocarbon in all samples (*N*=30)

[d] Values represent the mean concentration of each hydrocarbon for *P. barbatus* foragers (*N*=50)

*Compounds tentatively identified based on mass spectral interpretation and prior literature

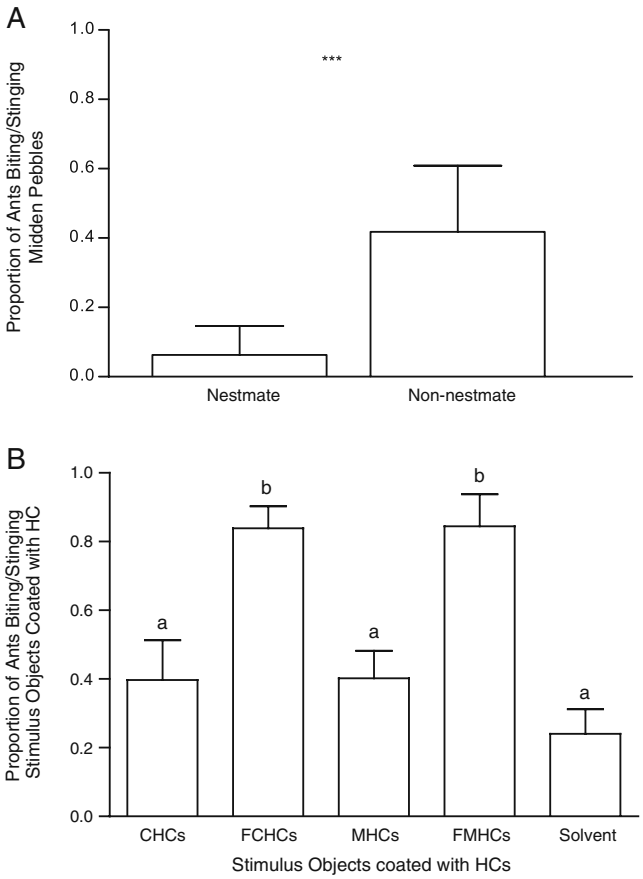


Fig. 3 **a** The proportion of workers that displayed agonistic behavior toward nestmate and non-nestmate midden pebbles. **b** The proportion of workers that displayed agonistic behavior toward glass blocks treated with nestmate or non-nestmate hydrocarbons (HCs) extracted from workers' cuticles or from midden pebbles. Each bar shows the mean proportion of ants that displayed biting or stinging behavior out of all ants that touched the stimulus objects. Lowercase letters indicate significant differences. Error bars represent the standard error of the mean. CHCs– cuticular HCs from nestmates; FCHCs– non-nestmate (foreign) cuticular HCs; MHCs– colony midden HCs; FMHCs– foreign midden HCs; Solvent –pentane

4), but there was no difference among treatments, and thus no effect of the zone from which Perlite was added (*ANOVA*: $F_{3,\ 16}$=1.498, P=0.2531). The average changes in forager speed to nest entrance (SD) for treatments 1–4 were 0.91 (0.12), 0.87 (0.12), 0.84 (0.12), and 1.03 (0.20), respectively.

The Perlite itself did not disrupt the foraging behavior of the ants. To determine that adding inert pebbles to the nest mound, by modifying the nest structure, did not itself affect the speed at which foragers return to the nest, we compared the speeds of returning foragers from colonies with midden pebbles ranging from 50–99% pebble cover to the speeds of returning foragers from colonies whose midden pebbles were removed. The two groups did not differ in the speed of returning foragers (*Unpaired t-test:* t_{262}=0.9241, P=0.356), indicating that the amount of midden pebbles on the nest mound does not affect forager return speed. In response to extraneous material on the nest mound, *P. barbatus* increase the numbers performing nest maintenance and decrease the numbers foraging (Gordon, 1986, 1987). Here, we saw no difference in the number of nest maintenance workers before and after treatment (*Mann–Whitney U test:* U=5841, N_1=100, N_2=120, P=0.727), and the proportion of colonies that did not forage did not differ before and after treatment (before treatment = 19%, after treatment = 19.1%).

Discussion

Our results show that colony-specific chemical cues on the midden pebbles of *P. barbatus* colonies help the foragers return to the nest more quickly. These cues on the midden pebbles are the same HCs that *P. barbatus* workers use in nestmate recognition (Fig. 2; see Wagner et al., 2000). Workers can distinguish their own colony's midden from that of other colonies, and can distinguish their own colony's midden HCs from the midden HCs of other colonies.

The concentration of HCs in midden pebbles and on the soil surface below the midden pebbles increases as a forager moves closer to the nest entrance (Fig. 4). A possible mechanism for the HC gradient is ant density. *P. barbatus* worker densities are greater per cm^2 nearer the nest entrance than away from the entrance (unpublished data). Therefore,

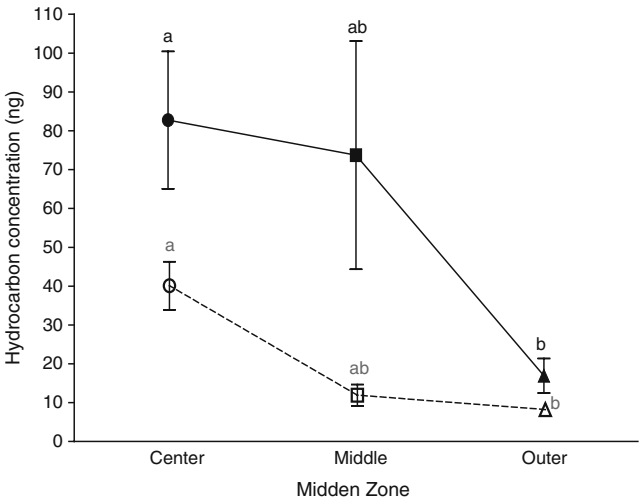


Fig. 4 Mean hydrocarbons in ng per 5 g of midden pebble samples and 2 g of soil samples for the center, middle, and outer midden pebble (closed shapes, solid line), and surface soil (open shapes, dashed line) zones. Center (*circle*), middle (*square*) and outer (*triangle*) zones as in Fig. 1. Lowercase letters above the shapes indicate significant differences. Error bars represent the standard error of the mean

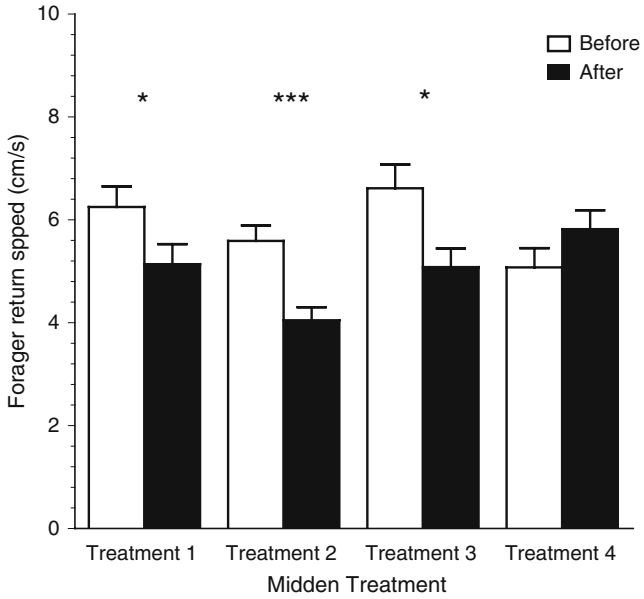


Fig. 5 Comparison of the mean forager return speed (cm/s) before and after treatment within each treatment group. Treatment 1: midden pebbles in the center and middle zones were diluted with Perlite. Treatment 2: midden pebbles in the outer zone were diluted with Perlite. Treatment 3: midden pebbles for all 3 zones were diluted with Perlite. Treatment 4: all midden pebbles were removed, leaving the bare soil that had been covered by midden material. *P*-values were obtained by comparing the square-root transformed forager return speeds before and after manipulation for each treatment group using unpaired t-tests. Error bars represent the standard error of the mean.* $P<0.05$, ** $P<0.01$, *** $P<0.001$

ant footfall or marking behavior occurs more in the center zones than the outer zones. The reduction in the concentration in the outer zones could be due to the difficulty in detecting HCs as their concentrations decrease or due to the greater volatility of certain HCs and the lack of sufficient ant footfall or marking to replace them.

Experimental manipulation of midden pebbles did not disrupt foraging, but it caused a decrease in the walking speed of returning foragers. When midden material was diluted with artificial pebbles lacking the colony-specific HCs, the speed of returning foragers decreased significantly. These effects did not vary according to the region of the nest mound on which the midden was diluted or removed.

The reduction in forager return speed is unlikely due to any physical or chemical properties of Perlite, which was of similar size and shape to the midden pebbles. If midden pebbles texture, size, shape, and/or color were significant factors in guiding ants to the nest, we should have seen a decrease, however slight, in the forager return speed in the removal treatment (treatment 4). Instead, there was a slight, although not statistically significant, increase in forager return speed after the removal treatment.

The rate at which inactive foragers leave the nest depends on the rate at which other foragers return with food (Gordon et al., 2008). Midden HCs influence forager return speeds, which influences forager return rates. Foraging activity affects food intake and interactions with neighbors, because encounters between foragers of neighboring colonies help to maintain foraging territory (Gordon, 1992). Thus chemical cues on the midden play a role in food intake, and in relations among conspecific neighbors.

We found a concentration gradient in HCs by zone on the midden, with a higher concentration toward the nest entrance, but our experiments did not elucidate the function of this gradient, at least in returning forager velocity. Our experiments compared the effect of disrupting the gradient by dividing the nest mound into three concentric zones. It may be that the gradient is important, but that the zones we chose arbitrarily do not correspond to areas that are functionally significant for the ants. Small gradients of HCs in the midden may be important, and finer scale manipulations are needed to investigate this. Perhaps, the chemical concentration gradient is used by *P. barbatus* foragers as a primary means of navigating the nest mound.

Chemical extractions from the top soil on the nest mound revealed the presence of a HC gradient on the soil as well as on the midden pebbles (Fig. 4). The presence of HCs, with a concentration gradient in the surface soil of nest mounds suggests that even in the absence of midden pebbles, there were sufficient HCs to be used by returning foragers. A colony usually stays in the same nest for its 20–30 year lifespan (Gordon, 1992). An accumulation of HCs in the midden and on the soil is not unexpected because of the stable nature of these compounds (Martin et al., 2009).

An interesting mechanism explaining the accumulation of HCs on the nest area as a form of colony recognition system was first reported for other harvester ants (see Grasso et al., 2005) whose fecal spots deposited on the nest surroundings contain colony-specific HCs. Interestingly these HCs are the same as those found on the cuticle of the ants. This mechanism also may be valid for *P. barbatus* and could explain the HC accumulation, not only on the pebbles, but also on the soil surface as a consequence of massive area marking with feces by the ants. Hydrocarbons may be deposited by footprints from glands in the legs of ants (Billen, 2009; Lenoir et al., 2009) or by the Dufour's gland located near the tip of the abdomen (Greene and Gordon, 2007). Another possible source of colony specific HCs may be the post-pharyngeal gland which, at least in some ant species, is clearly involved in the formation of the colony specific label (see for example Soroker and Hefetz, 2000). Further research is needed to determine the origin of midden HCs, and whether specific task groups deposit and maintain HCs in the midden.

We do not rule out the role of olfactory cues, apart from HCs, in foraging behavior. When experimentally offered unusually abundant seeds, *P. barbatus* scouts lay phero-

mone trails from the feeding site to the nest with poison gland secretions (Gordon, 1991; Hölldobler et al., 2001). However, although we did not interfere with any marking behavior on the foraging trail or anywhere outside the nest mound, disturbing the midden clearly affected the behavior of returning foragers. This means that short-term cues alone, if they are used at all, cannot be the only means of guiding returning foragers to the nest entrance.

Harvester ants use midden HCs, a chemical cue applied collectively, to aid in the return of foragers. Several ant species use pheromonal or environmental cues when returning to the nest (Cammaerts and Rachidi, 2009; Steck et al., 2009, 2010). Hydrocarbons, on the other hand, are more stable, are colony-specific, and are regularly transferred from ants to their surroundings. Nest mound HCs have been associated with territorial behavior in ants (Grasso et al., 2005; Lenoir et al., 2009). The use of nest mound cuticular HCs contributes to the regulation of foraging activity because it helps foraging harvester ants to return to the nest more quickly, and thus adds to the precision with which forager return rate measures the current availability of food.

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Density-Related Volatile Emissions and Responses in the Red Flour Beetle, *Tribolium castaneum*

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Abstract Intraspecific attraction depends both on the cues provided by the attracting individual and the response of the attracted individual. These attracting cues are related not only to current conditions, but also are a reflection of individual and population life history. These relationships were examined by placing red flour beetle, *Tribolium castaneum* (Herbst.), adults in flasks at increasing densities and monitoring the changes in volatile chemical emission over time. Only certain chemicals were quantified: methyl benzoquinone, ethyl benzoquinone and 4,8-dimethyldecanal, all of which are known to impact the biology of *T. castaneum*. The flasks were used as sources for both quantification of the chemicals and for bioassays. Additional bioassays were conducted with synthetic 4,8-dimethyldecanal, a known aggregation pheromone component, to evaluate attraction with respect to population density. *Tribolium castaneum* density affected both the release of volatile chemicals and the responses of conspecifics to those chemicals. The results indicated that while there were important effects of beetle density on chemical emission and response, none of the chemicals evaluated emerged as promising synergists to the current aggregation pheromone 4,8-dimethyldecanal. The benzoquinones released in response to stress and density acted as anti-aggregation pheromones along with their accepted defensive function.

Key Words Aggregation pheromone · Anti-aggregation behavior · Benzoquinone · Coleoptera · Density · 4,8-Dimethyldecanal · Gas-chromatography-mass spectrometry (GC-MS) · Gas chromatography-electroantennal detection (GC-EAD) · Red flour beetle · Tenebrionidae · *Tribolium castaneum*

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Introduction

Tribolium flour beetles (Coleoptera: Tenebrionidae) have long been used as models for studying inter- and intraspecific competition (Park, 1948, 1962). They are an ideal model of these relationships, because they can complete their life cycle in small laboratory containers with a flour and yeast mixture. They also are long-lived and tolerate a broad range of environmental conditions (Sokoloff, 1974). Stored products, like other poorly defended nutrient rich resources, are very attractive to a wide range of consumers, and thus competition is fierce. Selection favors those individuals that can quickly find, utilize, and successfully defend their claim to the resource (Ziegler, 1976). Once the resource is occupied, it is depleted by many different forces, environmental, microbial, as well as animal. Other selecting forces come into play on this depleted resource, selecting for different features in colonizers and causing the primary colonists to disperse or migrate to other resources (Dingle, 1972; Ziegler, 1976). The red flour beetle, *Tribolium castaneum* (Herbst.) is an excellent colonizer of cracked grains with a high reproductive rate and cannibalistic offspring, enabling both resource utilization and defense (Park, 1962).

Population density plays an important role in both the composition of chemicals emitted by certain insect species and also their response to those chemicals. There is a large body of information about how the *T. castaneum* population reacts to various stimuli (Sokoloff, 1974). The chemicals produced by *T. castaneum* and by the confused flour beetle,

T. confusum (du Val) include an aggregation pheromone, 4,8-dimethyldecanal (DMD), which is emitted by males and attracts both sexes (O'Ceallachain and Ryan, 1977; Suzuki, 1980); a defensive secretion containing methyl and ethyl-1,4-benzoquinones as toxicants (Wirtz et al., 1978); and 1-pentadecene as a solvent, spreading, absorption agent, and as a copulation pheromone in *T. confusum* (von Endt and Wheeler, 1971; Keville and Kannowski, 1975; Markarian et al., 1978). There are at least two isomers of DMD that are biologically active, with an 8:2 mixture of the 4*R*,8*R*- and 4*R*,8*S*- mimicking the aggregation pheromone of *T. castaneum* (Suzuki et al., 1984). Previous research has examined the relative emission of these chemical components related to density (Faustini and Burkholder, 1987), but the behavioral response and a more precise measure of emission over time have not been addressed.

Insect density can affect development, both in terms of physical crowding and also density dependent chemicals (Roth and Howland, 1941; Hodjat, 1970). *Tribolium* larvae exposed to the benzoquinones given off by adults, particularly at high densities, may develop abnormalities (Mondal, 1990). Thus, the need to disperse from a previously colonized medium is not only brought about by a decrease in the quality of diet, but also by the toxicity of benzoquinone secretions. Many insects change their behavior in response to chemical cues related to density. For example, the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae), releases an antiaggregation pheromone (verbenone) at high population densities that causes conspecifics to stop their mass attack of one tree and instead disperse to surrounding trees (Thatcher et al., 1980). Social insects such as honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), use density cues to manage brood rearing, caste development, and other colony and individual behaviors (Wright, 2009). These examples cover cues that are related to individuals or groups perceiving chemical or physical cues and then changing their own behaviors, but they do not address the question of how responses to chemical cues change with population density.

Although a number of studies have been conducted on the biological responses of *T. castaneum* to the aggregation pheromone or the defensive benzoquinones, few have assessed the interactions of the two classes of volatiles particularly with respect to effects of population density on biology. The central questions herein addressed were: What was the most attractive blend of chemical signals released by *T. castaneum* and under what conditions was it released? This research showed how behavioral responses and volatile chemical releases change with *T. castaneum* population density and the interaction between both the aggregation pheromone and the defensive benzoquinones. Behaviorally active chemicals that had not previously been described for *T. castaneum* were searched for, but none were found.

Methods and Materials

Insects Insects were reared in 0.95 l glass mason jars in an incubator at 27.7°C and 60% RH, each jar contained 200 g of diet that consisted of whole wheat flour, enriched white cornmeal, and brewers' yeast (1:1:0.4).

Volatile Collection The first exploratory volatile collection was from one of these colony jars at the end of a generation (ca. 3500 insects per jar, the end result of 200 adults added to 200 g flour, volatile pull conducted after 8 wk had passed). Headspace volatiles were pulled from above the diet in the jar described above that still contained beetles of mixed life stages. The collection method consisted of inserting a piece of Teflon tubing through each of two holes in the lid and sealing them in place with Teflon tape. The inflow tube was inserted nearly to the surface of the diet, but the vacuum tube was inserted only 2 cm into the jar, ensuring that air was circulated throughout the jar to maximize capture of volatiles from both the diet and the beetles. Teflon tubing was used for inflow outside the jar, and Tygon tubing was used for vacuum downstream from the filter. Airflow into and out of the jar was regulated by flow meters (Aalborg, Orangeburg, NY, USA) to maintain a constant flow rate of 40 ml/min. An inline filter packed with 30 mg Super Q (Alltech, Nicholasville, KY, USA) was attached between the Teflon outflow tube and the end of the vacuum tube outside the jar to capture volatile chemicals in the air stream. Volatiles were collected for 24 h to account for any circadian fluctuations.

For the density volatile collection experiments, adult beetles were collected from culture jars by sifting and placed in 250 ml Erlenmeyer flasks containing 10 g of flour. Three experiments were conducted. In the first, there were 10, 50, 250, 500, 750, or 1,250 beetles per flask, with 3 flasks (replicates) for each density. In the second, beetle density was 50, 500, or 1,250 beetles per flask, with six replicates per density, and in the third, there were 12 flasks, each with 1,250 beetles. In all three experiments, volatiles were collected with Teflon tubing inserted through the holes of a two-hole Neoprene stopper inserted into the neck of the flask. As before, the inflow tube extended nearly to the surface of the flour, and the vacuum tube extended only 2 cm into the flask, thus ensuring good mixing. Collection and extraction of volatiles was the same as described above. For the first set of densities, volatile chemicals were collected on days 2, 4, 7, and 10. For the second set,

volatiles were collected on days 2, 4, 9, and 22, and for the third set on days 2, 4, 7, 10, 12, 15, 19, 22, 24, 27, and 29.

Chemical Extraction and Analysis Chemicals were extracted from filters with 200 μl of methylene chloride (Sigma-Aldrich, St. Louis, MO, USA), after 5 μl of an internal standard (80 ng/μl of nonyl acetate (Sigma-Aldrich) in methylene chloride) had been added to the top of the packing material. Chemicals in the initial samples were preliminarily identified with GC-MS (Hewlett Packard HP6890 with HP5973 MS in EI mode with a 30 m x 0.25 mm i.d. HP1 capillary column with a 0.25 μm film of methyl silicone. The injector inlet temperature was held at 240°C with He carrier gas moving through the column at 30 cm/sec. The oven held an initial temperature of 35°C for 1 min followed by a 10°C/min temperature ramp to 230°C and then held for 5 min. The transfer line to the MS was kept at 240°C. Chemicals were then quantified by analysis with GC-FID (HP 6890 with the same column and parameters as above except the FID detector was held at 250°C). Standards for methyl- and ethyl-1,4-benzoquinones and 1-pentadecene were purchased (Sigma-Adrich), and DMD identification was confirmed by a volatile pull from an attractant septum (Trécé, Adair, OK, USA).

Gas Chromatography-Electroantennal Detection (GC-EAD) Analysis To determine which chemicals *T. castaneum* could detect from the old jar, we used coupled gas chromatography-electoantennographic detection (GC-EAD). One μl of the methylene chloride extract of the old jar headspace volatiles from Super Q was run on the GC (Hewlett Packard HP 6890 with an Alltech Econo-Cap EC-1 30 m× 0.25 mm capillary column with a 0.25 μm film of methyl silicone). The cool on column injection inlet was kept at a constant 220°C with a 40 cm/sec flow rate through the column, the FID detector was kept at 250°C, and the transfer line to the antenna at 230°C. The oven temperature started at 38°C for 1 min then increased at 10°C per min to 230°C before holding at that temperature for 4.5 min. For GC-EAD, both antennae were removed from two beetles with a razor blade, and each antenna was carefully placed across a gold plated electrode (Syntech, Netherlands) with both ends resting on a thin layer of electrode gel (Spectra 360, Parker Labs, Fairfield, NJ, USA). After each run, the electrodes were wiped clean and four new antennae were mounted for the next run. Five runs were conducted consecutively and none was discarded. Noise removal with a filter of 8 was used and the baseline calculated by the Syntech software (EAD 2000, Ver. 2.6) was used as the baseline.

Laboratory Walking Behavioral Assays For background response bioassays, males and females were sexed as pupae and then raised in 26 ml plastic vials with a small amount of flour. An oblong bioassay chamber (elliptical, 17.5 cm long, 0.95 cm high and 5 cm wide at the widest point) was used for all two-choice bioassays (Duehl et al., 2011). Insects were introduced into a chamber that connected between a vacuum manifold and the center of the arena, and air was pushed in at the thinnest points at either end of the ellipse. Sexed beetles were added to the chamber at least 12 h before the bioassays were conducted. One inlet of the bioassay chamber was clean air and the other was air passing through a jar containing old diet and beetles as described above. The rearing jar functioned better than a sealed volatile collection chamber because beetles and diet were not disturbed by a transfer. Six trials for males and 9 trials for females were conducted with 8 beetles per trial, and each trial gave the beetles 5 min to respond to the volatiles by moving from the introduction chamber to one of the ends of the ellipse. An additional set of 6 trials per sex was run with older (53 d), more conditioned diet that had originally contained 10 g of flour and 1,250 adult beetles as a volatile source. Many of the beetles in this colony had died, and the surface of the diet had taken on a blackened color.

An additional set of three separate bioassay-based experiments were conducted to evaluate the responses of *T. castaneum* individuals to density related cues. The first two assays used the oblong arena; eight individuals per replicate were removed from a flask (with a specific population density) with an air pencil and placed in the insect introduction chamber without diet overnight (about 16 h) to calm (Duehl et al., 2011). Bioassay 1 (beetles to other beetles): assays were conducted to determine the behavioral response of beetles to volatiles from flasks containing different densities of beetles (10, 50, 250, and 1,250 individuals). Each flask was set up as the volatile source and tested against beetles from each of the other densities but not the same density. Bioassay 2 (beetles to attractant): with beetles from flask densities of 50, 500, and 1,250, assays were conducted to examine response to volatiles from commercial attractant septa (Trécé RFB/CFB attractant). The attractant septum was placed in a glass volatile collection chamber (Analytical Research Systems, Gainesville, FL, USA), and air was passed through the chamber at the same volume that it passed through the flasks in the previous bioassay. Bioassay 3 (beetles to attractant): a beetle was released onto a 53 micron opening nitex (monofilament nylon mesh) sheet stretched over two small Petri dishes, one containing a DMD (Trece; 1:1 4*R*,8*R*- and 4*R*,8 *S*-DMD) septum and the other empty. Ethovision XT (Noldus, Leesburg, VA,USA) tracked the movement of individual beetles (Duehl et al., 2011). The movements of 8 beetles from the 50, 500, and 1,250 density cultures were recorded for 10 min per individual, and the

time spent in the attractant and control zones was examined. The movements of beetles from the 50 density culture were the most variable and so an additional 11 assays were conducted.

Statistical Analyses All test statistics were calculated in SAS (SAS 9.2, Cary, NC,USA) and significance was judged at α=0.05. ANOVAs were conducted with PROC GLM; series were analyzed with PROC MIXED; and two-way comparisons were made with PROC TTEST, whereas correlations were calculated with Pearson's correlation coefficient in PROC CORR.

Results

The background response bioassays showed that volatiles released from the old jar were repellent (T_{15}=−6.09, P< 0.001) and that the repellency did not vary with sex of the responder (P=0.999) (Table 1). Additional bioassays were conducted with conditioned diet, and beetles that had been confined longer with less diet per beetle and odors from that material showed even greater repellence than did the rearing jar (T_{27} =−4.42, P<0.001). The relationships by sex for both old and older jars showed that both sexes were repelled by volatiles from those jars (Table 1). The decrease in response rate with jar age likely was related to beetles not moving upwind in response to the high levels of repellent compounds. Coupled GC-EAD demonstrated that the antennae of *T. castaneum* responded to the methyl- and ethyl-1,4-benzoquinones and the aggregation pheromone, DMD (Fig. 1). The magnitude of the responses to the chemicals differed significantly (P<0.05, N=5).

Conditioned medium clearly was repellent even though it contained a detectable level of aggregation pheromone. None of the volatiles from the densities examined produced significant attraction in bioassays (Table 2), but some high density cultures were significantly repellent, as indicated by *t*-test. Analyses of volatiles from jars with differing beetle densities showed that only the jar with the highest density differed significantly in the amount of volatiles released from the others (Fig. 2). Because of the variability among samples, the low, middle, and high densities were replicated, but only the highest density showed significant differences (Fig. 2). Although there was variability among jars and collections, beetles in bioassays did not show clear attraction to any beetle density. So while the aggregation pheromone was emitted by all flasks at levels detectable by GC and beetles, individuals in bioassays did not show attraction as they did to the DMD attractant.

Beetles taken from cultures of variable density and exposed to other culture densities, showed no significant attraction to any of the other culture densities and had significant repellence from the high density culture, similar to the response from the old rearing jar (Table 2). This led to assays to test the response of beetles from different density cultures to the commercially packaged aggregation pheromone. These assays showed that the attraction response was not related to the density of beetles in the source culture, although the Ethovision assay showed all were attracted to the septa (T_{34}=−5.93, P<0.001). The results by time spent in DMD zone were; 50 beetle density 77.9±18.8 sec, 500 beetle density 60.9±18.2 sec, 1250 beetle density 103.5±23.3 sec, while the total mean for the control was 9.48±1.18 sec. The results from both the two-choice test ($F_{2,72}$=0.12, P=0.89) and Ethovision non-contact bioassay ($F_{2,35}$=0.79, P=0.46) showed no significant differences between the responses of beetles from different densities to the aggregation pheromone.

Behavioral response across culture density was important but so too was an understanding of volatile emission patterns over time. The chemical emission rates quantified were those of chemicals detected in GC-EAD and also the other major component, 1-pentadecene. Volatiles collected from beetles at different densities after 2 d on fresh diet showed that the total amount of aggregation pheromone released reached a peak at about 500 beetles (Fig. 2). Benzoquinone emission increased as the number of individuals increased, but not linearly over time. Additional volatile collections on days 4 and 9, as well as a second set on the densities 50, 500, and 1,250, showed that by day 9 the benzoquinones increased while the amount of aggregation pheromone decreased. The analysis then was extended over a longer time period, first with the three

Table 1 Walking responses of *Tribolium castaneum* adults to volatiles from high density culture jars in an oblong bioassay arena

Sex	*N*	Culture	Towards Jar	Towards Control	Non-Responders
Male	6	High Density	1.2 ±0.5	5.7±0.4 [a]	1.1±0.5
Female	9	High Density	2.2±0.5	5.4±0.5 [a]	0.5±0.2
Male	6	Older HD	0.0±0.0	5.8±0.5 [a]	2.2±0.5
Female	6	Older HD	0.3±0.2	5.2±0.5 [a]	2.5±0.7

[a] Significantly different by a *t*-test comparison of the responses to the jars and the control (P<0.001),

when sexes were pooled by density challenge the responses towards the different cultures were also significantly different (t_{27}=−4.42, P<0.001).

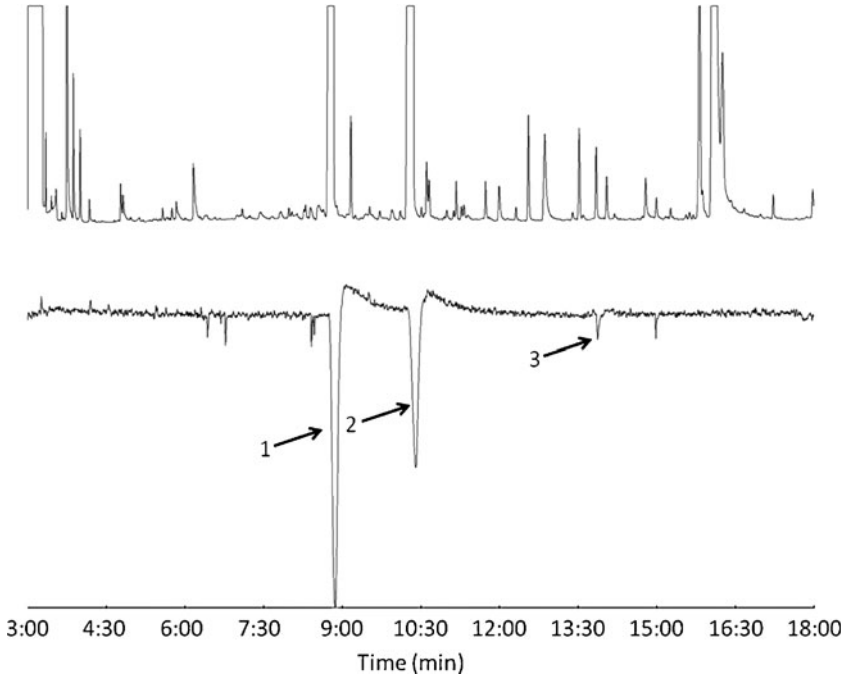


Fig. 1 GC-EAD recordings from unsexed *Tribolium castaneum* antennae to the volatile collection of the headspace of a *T. castaneum* infested culture medium. The bottom line represents the antennal response and the top line is the FID chromatogram. There are three important peaks, listed here with their average electrantennographic response: they correspond to 1, the methyl-1,4-benzoquinone (8.9±1.5 mV); 2, the ethyl-1,4-benzoquinone (5.2±0.7 mV); and 3, the aggregation pheromone 4,8-dimethyldecanal (1.0±0.1 mV). Each chemical elicited a significantly different electrical response, PROC GLM SAS α=0.05, N=5

densities to 22 d, and then with only one density, 1,250 beetles, to 29 d, to see what effect time would have on volatile emission (Fig. 3).

The last experiment examined volatiles from flasks with 1,250 beetles and 10 g flour over a month. It showed an initial increase in benzoquinone emission between introduction and 7 d, followed by 2 wk of decreased emission and then a final increase leading to the death of many beetles (Fig. 3). The methyl and ethyl benzoquinones were highly correlated throughout time (Pearson's correlation coefficient = 0.95, N=115, P<0.001); 1-pentadecene also was highly correlated with the two benzoquinones (Pearson's=0.83 and 0.87, respectively, P<0.001); while DMD was weakly negatively correlated with all three although not significantly correlated with 1-pentadecene (Pearson's= −0.20, -0.19, -0.16, P=0.032, 0.047, 0.092). Very few larvae were in the flasks during the experiment due to the high number of cannibalistic adult beetles. We sifted the flour in half of the flasks every week to remove larvae and found larvae only seven times, all after the third and fourth weeks, and only 1–10 larvae per flask.

Discussion

In these experiments, when *T. castaneum* adults from populations at different densities were exposed to the

Table 2 Walking responses of *Tribolium castaneum* adults from culture flasks at different densities to volatiles from culture flasks of increasing densities

Density	Volatile Source	Response			P-value[b]
		Control	Flask	NR[a]	
50	10	6.8±0.7	2.5±0.6	0.5±0.2	P<0.001
250	10	5.0±0.7	4.2±0.6	0.7±0.3	NS[c]
1250	10	5.8±0.6	2.7±0.4	1.5±0.5	NS
10	50	2.7±0.7	3.7±1.3	3.7±0.7	NS
250	50	5.2±0.7	3.7±0.8	1.2±0.5	NS
1250	50	4.2±0.9	3.5±0.6	2.5±0.4	NS
10	250	5.3±0.7	1.7±1.2	3.0±1.7	NS
50	250	6.7±0.7	1.8±0.5	1.5±0.6	P<0.001
1250	250	4.2±0.9	3.5±0.6	2.5±0.4	P<0.001
10	1250	5.7±0.9	1.7±1.2	2.7±0.7	NS
50	1250	7.7±0.6	0.8±0.5	1.5±0.4	P<0.001
250	1250	5.0±0.4	0.8±0.3	4.2±0.5	P<0.01

[a] NR = no response.

[b] Statistical comparisons were made between responses by beetles from known density containers to control clean air and a flask with beetles at the density listed under volatile source. Significance calculated in SAS with PROC GLM, N=6.

[c] NS = comparison not significant.

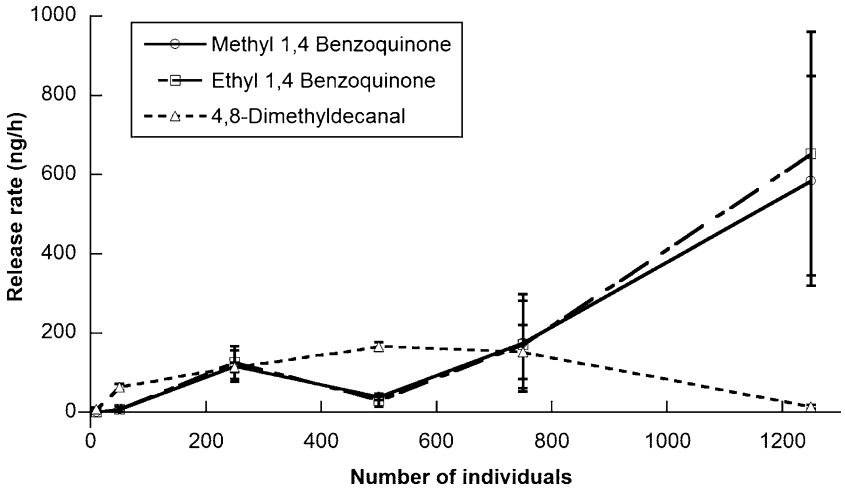


Fig. 2 The release rates of chemicals after each number of individual *Tribolium castaneum* had been in a 250 ml flask with 10 g flour for 2 d. The two benzoquinones increase along with the number of individuals, whereas the 4,8-dimethyldecanal production is maximized around 500 individuals

volatile compounds released by conspecifics held at different densities, no significant attraction was observed. Males alone in clean diet, however, are significantly attractive (Ryan and O'Ceallachain, 1976). We showed significant repellence and determined that at the highest density (1,250 beetles in 10 g of flour), the beetles consistently emitted volatiles that were repellant in our assays. This may be correlated with the release of very high levels of the benzoquinones that are repellant to larvae and adults at high concentrations (Mondal, 1985; Verheggen et al., 2007) or to the reduced release of the aggregation pheromone DMD (Fig. 2). The results of the bioassays showed no relationship between flask source density and the response to DMD. Thus, source colony density seems not to play a role in the response of individuals to DMD. As such, the repellency of the volatiles from high density colonies probably results from the high concentrations of the benzoquinones (ca. 600 ng/h of each) released at the 1,250 beetle density. Our analysis across beetle densities found benzoquinones were a consistent component of the volatile blend, and this may explain why beetles were not attracted in any of our studies using naturally released volatiles. This indicates that benzoquinones counteract the attraction of DMD at densities as low as 10 beetles/flask (Table 1) for *T. castaneum*. Nonetheless, DMD is clearly an aggregation pheromone, and our results suggest that it may act as a marker signifying good conditions as opposed to functioning only as an aggregation pheromone. In this scenario, beetles dispersing from high density cultures would search for fresh flour, and then indicate that they found a good location by releasing more of the DMD.

These responses to chemical stimuli by *T. castaneum* fit with their ecological niche. *Tribolium castaneum* is a better early colonizer of stored products than *T. confusum*; the latter are more attracted to and competitive in conditioned medium (Park, 1948; Ghent, 1963). Since *T. castaneum* has the competitive advantage in clean flour, it would avoid higher benzoquinone concentrations, basing attraction on DMD. Thus, while DMD is the commonality in the attraction of the two most pestiferous *Tribolium* species,

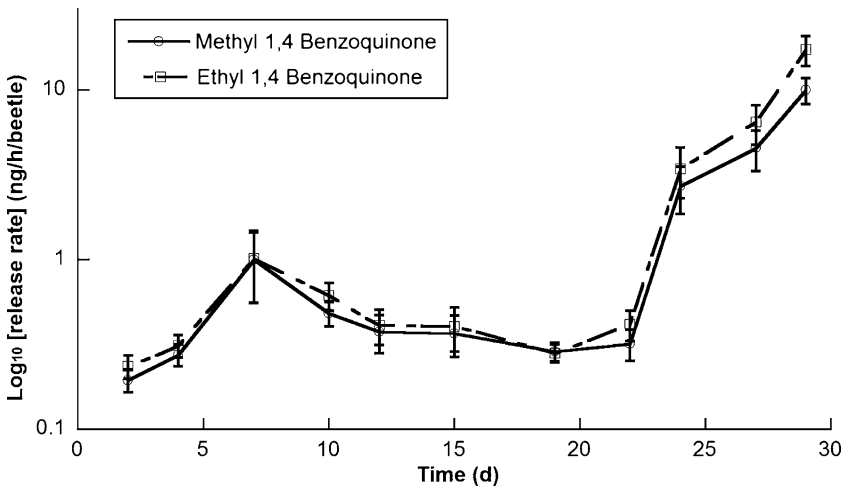


Fig. 3 Levels of methyl- and ethyl-1,4-benzoquinone released by 1,250 *Tribolium castaneum* in a 250 ml flask containing 10 g of flour. The benzoquinone levels increased for the first week; decreased for the next two weeks; and then increased to very high levels that killed many of the beetles in the flask. The axis scale is $\log_{10}$ to best display the change over time

the addition of benzoquinones as a synergist (Verheggen et al., 2007) most likely would increase attractant specificity and exclude *T. castaneum*. The bioassay results described here looked only at walking movement in response to volatile cues. Over flour, density cues both direct movement into the medium and also away from it (Ghent, 1963). Translating these results to a large three dimensional flour environment could mean beetles moving away from high density areas but staying in the flour or moving to the surface and dispersing.

A thorough examination of the volatile chemicals released over time by *T. castaneum* from cultures at different densities indicated that both exposure time and density strongly influenced the relative composition of the chemical blend. The total amount of DMD was greatest at intermediate densities, 500 individuals in 10 g of flour, although individual emission was greatest at the lowest densities. Levels of constitutive benzoquinones in *T. castaneum* increase during the first 30 d of adult life and then remain constant (Wirtz et al., 1978; Unruh et al., 1998). Therefore, the observed pattern of volatile emission was surprising; benzoquinones increased for the first week (Faustini and Burkholder, 1987) but then decreased for the following two weeks, before increasing exponentially. An explanation for this pattern should be sought through further experimentation, but the pattern was not related to immature individuals in the diet and did not match the amount of benzoquinones sequestered inside the beetles (Unruh et al., 1998). We suggest that the initial emission peak may be related to dispersal and the following decrease to the acceptance of the location and high density. The final exponential increase in volatile emission needs to be further investigated. An examination of the quality of the diet could show if toxicity leads to a new impetus for dispersal and stimulates additional benzoquinone release. The latter assertion is supported here by the death of 690±103 of the original 1,250 beetles per flask by the end of the experiment, with very few dying before the last few days.

This research indicated that none of the chemicals released by *T. castaneum* was likely to synergize DMD attraction. Additionally, the benzoquiones released under high density and stress functioned as antiaggregation cues, with high levels representing strong repellence. Low levels of benzoquinones were consistently released and were detected in all samples across densities; intermediate levels indicate antiaggregation, and very high levels seem correlated with resource exhaustion and mortality. The constitutive internal concentrations that increase during their youth (Wirtz et al., 1978; Unruh et al., 1998) did not correspond to the volatile levels observed, and they probably serve a defensive function (Sokoloff 1974). Future searches for attractants should focus on cues other than intra-specific attraction, instead looking to food and environmental cues.

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Sequestration of Camptothecin, an Anticancer Alkaloid, by Chrysomelid Beetles

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Abstract Camptothecin (CPT), a monoterpene indole alkaloid, is a potent inhibitor of eukaryotic toposiomerase-I. Several derivatives of CPT are in clinical use against ovarian and lung cancers. CPT has been reported from several plant species belonging to the order Asterids, with the highest concentration in *Nothapodytes nimmoniana* (family Icacinaceae). In this paper, we report an intriguing observation of chrysomelid beetles *(Kanarella unicolor* Jacobby) feeding on the leaves of *N. nimmoniana* without any apparent adverse effect. LC-MS/MS analysis of the beetles indicated that 54.9% of the ingested CPT's was recovered from the wings, followed by lesser amounts in the head and abdomen. LC-HRMS analysis revealed that most of the CPT in the insect body was in the parental form available in the plants without any major metabolizable products, including sulfated and glucuronilated forms. The mechanism by which the beetles are able to tolerate substantially high levels of CPT in their body tissue is under investigation.

Key Words Sequestration · Anti-cancer · Alkaloid · Camptothecin · Chrysomelid beetles · Topoisomerase I

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Introduction

Camptothecin (CPT), a monoterpene indole alkaloid, is one of the most potent cytotoxic compounds (Hsiang et al., 1985; Pommier, 2006). It is toxic to most eukaryotes including insects such as termites (Li, 2002) and drosophila (Cunha et al., 2002). CPT inhibits the enzyme topoisomerase I, which is required in DNA replication and transcription (Hsiang et al., 1985). Because of this property, several derivatives of CPT are in clinical use against ovarian, small lung and refractory ovarian cancers (Pommier, 2006). CPT is naturally distributed in several plant families of the order Asterids (Larsson, 2007). Among these, leaves and stem bark of *Nothapodytes nimmoniana* (Grahm) Mabb. (Icacinaceae), a small tree in southern India, accumulate the highest content of CPT (0.3–0.5%) (Ramesha et al., 2008). In this paper, we report an intriguing observation of specialist chrysomelid beetles (*Kanarella unicolor* Jacobby) that feed on leaves of *N. nimmoniana* without any apparent adverse effect. Liquid Chromatography-Mass Spectrometry-Mass Spectrometry

(LC-MS/MS) and Liquid Chromatography-High Resolution-Mass Spectrometry (LC-HRMS) analysis was performed to evaluate the fate of the ingested CPT in the insect body. We found that most of the ingested CPT is sequestered in the wings without any major metabolizable products. We discuss these results in the light of the possible mechanisms by which the beetles are able to tolerate substantially high levels of CPT in their body tissue.

Methods and Materials

Sampling and Maintenance of Adult Beetles We collected chrysomelid beetles feeding on leaves of *N. nimmoniana* from Biligiri Rangaswamy Temple (BRT) Wildlife Sanctuary (11.40° to 12.09° N and 77.05° to 77.15° E) in the state of Karnataka, India. The beetles were brought to the laboratory, School of Ecology and Conservation, University of Agricultural Sciences, Bangalore, India and maintained in culture jars on excised leaves of *N. nimmoniana*. Leaves were replaced every day. Adults fed actively under captivity. Throughout the culture period, the pellets (excreta) were collected. Beetles also laid eggs during this period (some of these eggs failed to hatch even after several months of incubation and hence were considered 'sterile'). These also were collected. Voucher specimens of the beetle (*Kanarella unicolor* Jacobby, subfamily Galerucinae, tribe Luperini, order Coleoptera) have been deposited in the Insect Museum, Department of Entomology, University of Agricultural Sciences, Bangalore, India.

Extraction of Camptothecins At the end of 25 days of culture, beetles (N=33) were collected, pooled, and processed for analysis of CPT and related compounds. Beetles were dissected into different parts; head, abdomen (including legs), and wings (hind and fore wings). While separating the abdomen, care was taken to ensure removal of undigested leaf material in the gut to avoid contamination of CPT from the host tissue. Insect pellets and sterile eggs collected over the culture period also were used for chemical analysis. The respective body parts, pooled from the 33 beetles, were ground in a pestle and mortar, and resultant fine powder was extracted for camptothecins. A known amount of tissue powder from each of the samples was extracted in 10 ml of 80% ethanol at 60°C for 3 hr in a shaking water bath. After cooling to room temperature, the extract was centrifuged at 10,000 rpm for 10 min at 10°C (Hermle, Germany). The supernatant was passed through a 0.2 μ filter (Tarsons, India) and analyzed for camptothecins.

LC-MS/MS Quantification of Camptothecins LC-MS/MS analysis of the extracts was performed using a Luna C18 column (0.3×150 mm, 3 μm Phenomenex, Torrance, CA, USA), with a mobile phase of H_2O with 10 mM NH_4OAc (solvent A) and CH_3CN with 0.1% HCO_2H (solvent B). All separations were performed at 30°C. NH_4OAc, HCO_2H, CH_3CN (Merck, Germany) and ultra-pure H_2O (Millipore, Schwalbach, Germany) were HPLC grade. Gradient elution consisted of the following ratios of solvent A/solvent B: 95:5, 48:52 and 0:100, with a total run time of 12 min. The column was then returned to the initial condition of 95:5 over about 0.5 min. After each run the column was re-equilibrated for 2 min. The flow rate was 0.3 ml/min with an injection volume of 10 μl.

Selected reaction monitoring (SRM) was performed using a highly sensitive TSQ quantum ultra AM mass spectrometer (Thermo, Finnigan) equipped with an ESI ion source (Ion Max) operating in positive mode. Nitrogen was employed as both the drying and nebulizer gas. Each mass transition was monitored at a peak width of 1 and a dwell time of 0.3 sec. External calibration was performed with the authentic standards in the concentration range of 0.005-10 μg/ml for CPT and 10-OH-CPT, and 0.005-1 μg/ml for 9-MeO-CPT. Limits of detection (LOD) for CPT, 10-OH-CPT and 9-MeO-CPT were 10, 5, and 4 ng/ml, respectively, providing further evidence of the high sensitivity of the system.

LC-HRMS Identification of Carboxylate, Glucuronilated and Sulfated Forms of Camptothecins Carboxylate, glucuronilated, and sulfated forms of CPT were identified based on their exact mass and retention times in LC-HRMS. Exact mass of all these CPT forms were calculated based on the molecular formula of the respective derivatives. The spectrometer was equipped with a Dionex micro-HPLC system Ultimate 3000 consisting of pump, flow manager and auto sampler with an injection volume of 0.6 μl. Nitrogen was used as sheath gas (6 arbitrary units), and helium served as the collision gas. Separations were performed by using a C18 column (0.3×150 mm, 3 μm, Phenomenex Gemini Torrance, CA, USA) with mobile phase H_2O containing 0.1% HCO_2H (solvent A) and CH_3CN with 0.1% HCO_2H (solvent B) and with a flow rate of 4 μl/min. Samples were analyzed using a gradient program with the following ratios of solvent A/solvent B: 95:5, 70:30, 30:70, 0:100, with run times of 0–5, 5–12, 12–24, 24–30 min, respectively. After 100% B, the system returned to its initial condition of 95% A within 1 min. After each run, the system was re-calibrated for 6 min. Owing to the relatively high solubility of carboxylate, glucuronilated and sulfated forms of CPT compared to parental CPT, we expected the elution of these forms to be much earlier than CPT or 10-OH-CPT.

The spectrometer was operated in positive mode with nominal mass resolving power of 60000 at *m/z* 400 (1 spectrum/*s* and with a mass range of 200–800). The scan rate



Fig. 1 Chrysomelid beetle feeding on *Nothapodytes nimmoniana* leaf

was 1 Hz with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation. Bis-(2-ethylhexyl)-phthalate (DEHP) was used as internal lock mass of *m/z* 391.28429.

Results and Discussion

During an extensive survey of the natural populations of *Nothapodytes nimmoniana* in its distributional range (8° to 15° N) in southern India, we found a fairly consistent occurrence of chrysomelid beetles (*Kanarella unicolor* Jacobby, subfamily Galerucinae, tribe Luperini, order Coleoptera) feeding on the leaves (Fig. 1). Only adult beetles were found on the plant; we did not recover larvae and pupa. The adults feed exclusively on *N. nimmoniana* by making small perforations on the leaf lamina. Beetles

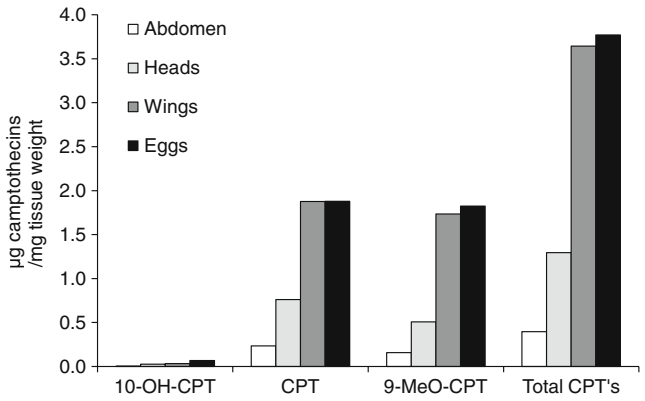


Fig. 3 Concentration of CPT and its metabolites in different body parts of beetles as quantified by LC-MS/MS. All data refer to a single analysis of all beetles (N=33) pooled

fed randomly on leaves irrespective of the leaf age and their CPT content (supplementary material Fig. S1).

For a set of laboratory maintained beetles that were fed exclusively on *N. nimmoniana* leaves for 25 days, we examined the relative proportions of CPT and related compounds in different body parts. LC-MS/MS of samples revealed the presence of CPT in head, abdomen, wings (both hind and fore wings) and also in the eggs laid by the beetles (Fig. 2). Other natural derivatives of CPT such as 10-hydroxy camptothecin (10-OH-CPT) and 9-methoxy camptothecin (9-MeO-CPT) that are known to be present in host leaves also were detected. Concentration of CPT was highest in wings (1.87 µg/mg-tissue weight), followed by head (0.76 µg/mg-tissue weight), and least in the abdomen (0.23 µg/mg-tissue weight). A similar pattern also was observed for 10-OH-CPT and 9-MeO-CPT, except the concentration of 10-OH-CPT was 10–15 fold less compared to other two compounds. In some samples, its concentration was negligible (Fig. 3).

We also examined whether or not the beetles metabolize CPT into its inactive forms, the carboxylate, glucuronilated or sulfated forms. None of the sulfated and glucuronilated forms

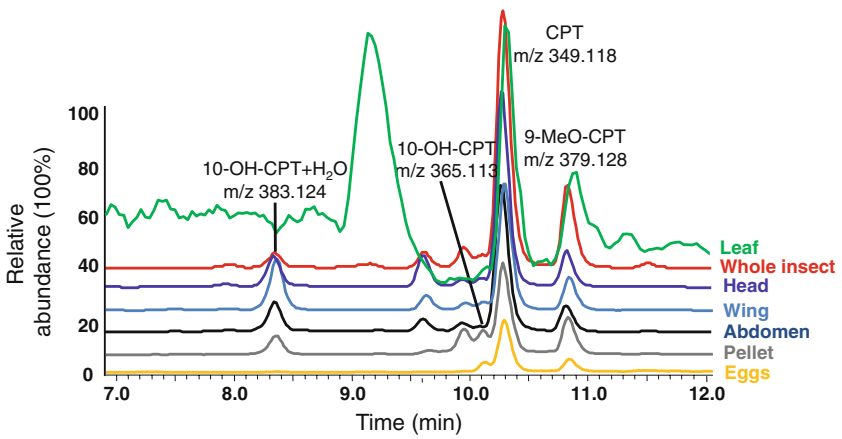


Fig. 2 Extracted total ion chromatograms (TIC) of *N. nimmoniana* leaf and different body extracts of beetle including eggs and pellets. TIC's of different samples were overlaid on each other and hence, except TIC of eggs, TIC's of other extracts does not start from 0% ionic abundance. Intensity of the peaks does not correlate with concentration of metabolites in different body parts. The large peak at ~9 min in the leaf sample is n-butyl benzenesulfoamide, plasticizer ($[M+H]^+$ 214.08963)

of CPT was detected in any of the body parts, when analyzed for their exact mass traces in LC-HRMS; sulfated CPT (cald; m/z 429.0750), sulfated 10-OH-CPT (cald; m/z 445.072), sulfated 9-MeO-CPT (cald; m/z 459.085), glucuronilated CPT (cald; m/z 525.152), glucuronilated 10-OH-CPT (cald; m/z 541.147), glucuronilated 9-MeO-CPT (cald; m/z 555.162). However, we did recover the carboxylate form of 10-OH-CPT (Rt 8.3 min, m/z 383.124) (Supplementary material Fig. S2) in all body parts including the pellets. The low concentration of carboxylate form of 10-OH-CPT (based on intensity in TIC spectra) compared to its parental form, however, suggests that the insects do not metabolize CPT to any substantial degree (Fig. 2).

In summary, the highest concentration of CPT and related compounds (10-OH-CPT and 9-MeO-CPT) was located in the wings (3.64 μg/mg-tissue weight amounting to 54% of total body CPTs), followed by abdomen (0.39 μg/mg-tissue weight, amounting to 28%), and head (1.29 μg/mg-tissue, amounting to 18%). On a single beetle body weight basis, disregarding the loss of CPT through pellets, the beetles accumulated about 3.02 μg of CPT at any given point of time, much higher than 1.7 μg/larvae that are lethal to Drosophila larvae (Cunha et al., 2002).

Sequestration of plant secondary metabolites, as predator deterrence behavior is well documented in insects, including beetles (Hartmann et al., 2001; Willinger and Dobler, 2001). A number of insects have developed adaptations to sequester pyrrolizidine alkaloids from their host plants and utilize them against their own predators (Hartmann et al., 2001). Frequently, the ingested plant secondary metabolite are either metabolized into products that are not detrimental to the insects or sequestered from the haemolymph into exocrine glands or into metabolically poor tissues such as the pronotum and elytra (Aregullin and Rodriguez, 2003); the latter serves as a "defense shield" against predators. Our results are the first to report the sequestration of CPT, a cytotoxic compounds by beetles. As indicated,, most of the CPT is sequestered in the parental chemical form in the elytra, without any major chemical conversion or metabolism. While we have not yet established the evolutionary significance of such sequestration, it is likely that the beetles use this compound to deter predators (Schroeder et al., 2006; Despres et al., 2007). It would be interesting to examine the mechanism through which the beetles tolerate high levels of CPT. Recently, Sirikantaramas et al. (2008) showed that plant species that produce CPT have specific mutations in their topoisomerase I that make these enzymes resistant to CPT. We are currently investigating if beetles have such mutations in their topoisomerase I.

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Lysophospholipids in the Mediterranean Sponge *Oscarella tuberculata*: Seasonal Variability and Putative Biological Role

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Abstract Lysophospholipids (LPLs) are recognized as important signaling molecules in metazoan cells. LPLs seem to be widely distributed among marine invertebrates, but their physiological role remains poorly known. Marine sponges produce original phospholipids and LPLs whose isolation and structural elucidation rarely have been reported. Two LPLs were isolated for the first time from the Mediterranean Homoscleromorph sponge *Oscarella tuberculata*: a bioactive lyso-PAF already identified in some other sponge species; and the new lysophosphatidylethanolamine C20:2 (LPE 1). The expression of LPL metabolites was investigated over time to determine their baseline variations and to relate them to the sponge reproduction pattern in order to better understand their putative role in the sponge life cycle. Expression levels of both compounds appeared to be highly correlated displaying significant seasonal fluctuations with maximal values in summer and minimal in winter. A significant higher LPL content was detected in reproductive sponges and especially in females, with a peak occurring during embryogenesis and larval development. The results suggest that LPLs could play a role of mediators in sponge embryogenesis and morphogenesis.

Key Words Lysophospholipids · Homoscleromorpha · Natural variability · Reproductive cycle

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Introduction

Phospholipids (PLs) are essential constituents of cell membranes, but their importance in living organisms is not restricted to this role (Vance, 2002; D'Arrigo and Servi, 2010). Among them, lysophospholipids (LPLs) represent only 0.5 to 6% of the whole PL content of cell membranes (Birgbauer and Chun, 2006; Torkhovskaya et al., 2007), their structural role being considered minor. However, they are recognized as second messenger molecules in cellular signal transduction processes (Torkhovskaya et al., 2007; D'Arrigo and Servi, 2010). These molecules exhibit a wide range of biological activities, and are among the most interesting lipids for pharmaceutical applications (Moolenaar, 2000; Birgbauer and Chun, 2006). For example, LPL receptors are required for the proper development and functioning of cardiovascular, nervous, immune, respiratory, and reproductive systems in mammals (Rivera and Chun, 2008). They also seem to play a critical role in pathological processes such as inflammatory reactions, autoimmune diseases, cancer, and atherosclerosis (Mills and Moolenaar, 2003; Gardell et al., 2006). The best known lysophospholipid messenger is a naturally occurring "platelet activating factor" (PAF or 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine), which mediates cellular interactions through a membrane-

bound G-glycoprotein receptor (Snyder, 1995; Prescott et al., 2000). PAF and its analogues were first identified as defense molecules involved in pathological processes such as anaphylaxis and inflammatory reactions (Archer et al., 1985; Vadas et al., 2008). Among the analogues, Lyso-PAF (2-*sn*-deacetyl) also exhibits cancerostatic properties (Mangold and Weber, 1987; Brachwitz and Vollgraf, 1995) and potent antimicrobial activity (Steel et al., 2002). Both PAF and lyso-PAF analogues are abundant in invertebrates and widespread among marine invertebrate metazoans, but their physiological roles remain to be defined (Sugiura et al., 1992).

Among aquatic invertebrates, sponges have the simplest body-plan adapted for filtrating suspended particles in the water column. Sponges produce a diversity of secondary metabolites that often have valuable biological activities (Sipkema et al., 2005). Marine sponge organic extracts contain a high proportion of lipids that may contribute to the entire bioactivity of the organism (Djerassi and Lam, 1991; Dembitsky et al., 2003). In general, sponge lipids differ from lipids of other metazoans in their chemical structure and biochemical properties (Djerassi and Lam, 1991). Sponges harbor a unique composition of membrane bilayer, where phospholipids containing very long alkyl chains (C_{24}-C_{38}), the so-called "demospongic fatty acids", coexist with a high proportion of unusual sterols (Djerassi and Lam, 1991). In addition to the usual ester-linked PLs, sponges produce ether-PLs such as lysophosphatidylcholines (LPCs), which have been reported for their cytotoxic and antimicrobial properties (Dembitsky et al., 1989; Dembitsky, 1996; Shin et al., 1999; Alam et al., 2001; Lee et al., 2003; Zhao et al., 2003; Müller et al., 2004; Mansoor et al., 2005).

After our work on the chemical diversity of Mediterranean Homoscleromorph sponges (Ivanišević et al., 2011), we focused attention on the species *Oscarella tuberculata*. In its environment, this thinly encrusting species has several colors (yellow to blue), has no known predator, and can cover in some places a high percentage (up to 100%) of the rocky substrate. It is able to overgrow other sessile invertebrates (sponges, gorgonians, and bryozoans), a particular habit that might be related to the production of efficient allelochemicals. *Oscarella tuberculata* previously has been reported to produce unusual lipids such as rare and long alkyl chain 5-substituted pyrrole-2-carbaldehydes and sterol endoperoxides (Cimino et al., 1975; Aiello et al., 1991; Loukaci et al., 2004). However, our metabolic fingerprinting approach indicated a larger chemical diversity than previously reported (Ivanišević et al., 2011). The same approach was used to evaluate the *O. tuberculata* intraspecific variability in secondary metabolite production on a large geographic scale (along the Mediterranean coast). No significant variation in metabolite composition was found in different color morphotypes, with the intraspecific variability being essentially quantitative, marked by variation in the expression level of major compounds (Ivanišević et al., 2011). Spatial and temporal variations in concentrations of major secondary metabolites have been documented previously in a few sponges, ascidians, and other sessile marine invertebrates (Page et al., 2005; Lopez-Legentil et al., 2006; Abdo et al., 2007). Long-term temporal variations often have been explained by ecological and environmental factors (Page et al., 2005; Abdo et al., 2007). However, the influence of primary biological functions has been rarely investigated to explain secondary metabolite variations (Lopez-Legentil et al., 2006). Production of secondary metabolites is energy consuming, however, it should require an optimized allocation of resources with respect to primary processes such as growth and reproduction (McClintock and Baker, 2001).

The primary aim of this study was to perform a chemical study of *O. tuberculata* in order to isolate and characterize the major non described metabolites. The second objective was to study the seasonal variability in the production of these metabolites, to relate it to seawater temperature fluctuations and to the sponge reproductive cycle. If LPLs have an ecological or physiological role, we would expect a pattern of variation of their expression level related to biotic or abiotic factors. On the other hand, if their role is mainly structural, only slight variation of their expression level over time with no special trend would be expected.

Methods and Materials

Biological Material Oscarella tuberculata (Schmidt 1868) (Fig. 1) is one of the most common Homoscleromorpha sponges in western coralligenous Mediterranean habitats. It is found in shallow waters, on rocky bottoms (coralligenous and semi-dark cave communities) from 4 to 35 m deep, and



Fig. 1 *In situ* picture of *Oscarella tuberculata* within the coralligenous community

from the Gibraltar Strait to the Eastern Mediterranean. The sponge is thinly encrusting with a lobate surface and thickness of 2–5 mm. Several color morphotypes have been observed *in situ*, varying from yellow, greenish to blue.

Sampling Procedure *Oscarella tuberculata* samples for metabolite isolation and structural elucidation were collected by SCUBA off the Marseille coast (France) in April 2008. A population monitoring for reproductive cycle and secondary metabolite production was performed between October 2001 and August 2003 (two annual cycles) at 13 m depth in Maire Island (Grotte à Corail, Marseilles). During this period, six different individuals per month of the yellow color-morph were randomly collected. A small part of every individual (about 1 mm^3) was cut off and fixed for histological analysis; the rest being immediately frozen at −20°C, freeze-dried, and conserved at −20°C for the chemical survey.

Reproductive Cycle Description To characterize the life cycle and assess reproductive effort, samples were fixed in Bouin fixative. Tissue fragments were dehydrated through an ethanol series, and embedded in paraffin. Serial sections of 6 μm in thickness were mounted on glass slides, stained with Trichrome of Masson and Goldner hematoxylin, and then observed under a light microscope. Digital pictures were captured in order to quantify the reproductive elements (spermatocyste, oocyte, embryo, and larva) using the ImageJ Software (http://rsb.info.nih.gov/ij/index.html). For each reproductive sponge, the reproductive effort was expressed as a percentage of sponge volume.

Chemical Equipment NMR experiments were performed on a 500 MHz spectrometer. Chemical shifts (δ in ppm) are referenced to the carbon (δ_C 49.0) and residual proton (δ_H 3.31) signals of CD_3OD, the solvent with multiplicity (s singlet, d doublet, t triplet, m multiplet). HPLC-MS analyses were performed using a VWR-Hitachi HPLC system and a Bruker Esquire 3000 Plus mass spectrometer equipped with electrospray ionization. Mass spectra were recorded in the positive mode. The HPLC system also was equipped with an autosampler (VWR-Hitachi L-2220), a Diode Array Detector (DAD, VWR-Hitachi L-2455), and an Evaporative Light-Scattering Detector (ELSD, Chromachem, Eurosep, France). High-resolution mass spectra (HRESIMS) were conducted on a LTQ Orbitrap mass spectrometer (Thermo Finnigan). HPLC purification were carried out on a Waters 600 system equipped with a Waters 717 plus autosampler, a Waters 996 photodiode array detector, and a Sedex 55 evaporative light scattering detector (Sedere, France). Gas Chromatography–Mass Spectrometry analyses were performed on a Hewlett-Packard HP-5890 chromatograph linked to an HP-5989-A mass spectrometer (70 eV) equipped with a HP-9000-345 integrator.

Extraction and Isolation of Major Compounds The lyophilized sponge (51.3 g) was extracted three times with $MeOH/CH_2Cl_2$ 1:1 (500 ml) at room temperature yielding 11.1 g of dry crude extract. The crude extract was fractionated by RP-C_{18} Vacuum Liquid Chromatography (elution with a decreasing polarity gradient from H_2O, H_2O/MeOH 1:1, H_2O/MeOH 1:3, MeOH, $MeOH/CH_2Cl_2$ 1:1 to CH_2Cl_2). The MeOH and $MeOH/CH_2Cl_2$ 1:1 fractions, showing similar HPLC profiles, were pooled and further fractionated by normal phase Diol flash chromatography (elution with a gradient of increasing polarity from Hexane/AcOEt 1:0 to 0:1, then AcOEt/MeOH 1:0 to 0:1). Control of the presence of phospholipid content was determined by TLC. The presence of the already known 5-alkylpyrrole-2-carbaldehydes was evidenced in the Hexane/AcOEt 9:1 fraction by using ^1H NMR and MS analyses. The AcOEt/MeOH 1:1 and MeOH fractions containing the phospholipids were pooled and subjected to C_6-phenyl semi-preparative HPLC (Phenomenex, Luna C_6-Phenyl, 250×10 mm, 5 μm) eluting with an isocratic MeOH/H_2O/Formic Acid 85:15:0.1 to afford pure lyso-PAF (1.5 mg) and LPE 1 (7 mg).

GC-MS Analysis of the Fatty Acid of LPE 1 A preparation of Fatty Acid Methyl Esters (FAMEs) and *N*-Acyl Pyrrolidides (NAPs) of LPE 1 (1 mg) was made prior to GC-MS analysis. FAMEs were obtained by methanolysis of LPE 1 (40 min under reflux in the presence of methanolic HCl 5%). NAPs were prepared by treatment of the resulted FAMEs with pyrrolidine/acetic acid (10:1, v/v) under reflux for 2 h. GC-MS analyses were obtained using a 30 m x 0.32 mm i.d. fused silica capillary column coated with DB-1 (0.25 μm phase thickness). The carrier gas was helium. Temperatures of the injector and the source were held at 240°C, and the temperature of the GC–MS interface at 290°C. The column temperature was programmed for FAMEs and NAPs from 180 to 310°C at 3°C/min. The fatty acid pyrrolidide of LPE 1 was identified at t_R 27.7 min (C20:2, Δ5,11).

Temporal Series Treatment by Metabolic Fingerprinting Freeze-dried samples were ground to obtain a homogenous powder, and 0.4±0.005 g of the powder was extracted in 5 ml of $MeOH/CH_2Cl_2$ 1:1 for 10 min in an ultrasonic bath. This operation was repeated three successive times. Filtrates of each extraction were combined and evaporated to dryness. Dry crude extracts then were dissolved in distillate water, and up to 2% acetone was added to complete the dissolution. Five ml of each dissolved extract

were loaded onto conditioned C_{18} SPE (Solid Phase Extraction) cartridges (0.2 g, Phenomenex Strata). After desalting with water, the organic extracts were eluted with 1 ml of MeOH/CH_2Cl_2 1:1. They were analyzed by HPLC-DAD-ELSD-MS. HPLC separation was achieved on a C_{18} column (Gemini, Phenomonex, 250×4.6 mm, 5 μm) using a linear gradient elution of H_2O/ACN/formic acid from 90:10:0.1 (isocratic from 0 to 5 min), to 0:100:0.1 (isocratic from 35 to 45 min, flow 1 ml/min). The column temperature was maintained at 30°C. The injection volume was set to 20 μl. The mass spectrometry detector (MSD) parameters were set as follows: nebulizer sheath gas, N_2 (50 psi); dry gas, N_2 (12 L/min); capillary temperature, 350°C; capillary voltage, 4000 V in negative ion ESI mode, 4000 V in positive ion ESI mode; lens 1 voltage, −5 V in (+) ESI; lens 2 voltage, −60 V in (+) ESI; isolation width for the MS experiments, *m/z* 1.0; collision gas, He; and collision energy, 35%. Data were collected between *m/z* 50 and 1200. The ELSD fingerprints were obtained after mobile phase nebulization at 38°C following evaporation at 50°C. The resulted metabolic fingerprints: ELSD and ESI-MS(+) (Base Peak Chromatogram) raw chromatogram data were processed by Data Analysis software. Filtering and baseline correction for ELSD chromatograms were defined by the following parameters: RT (Retention Time) range 0–50 min, Signal/Noise threshold≥5, Minimum relative area≥10% and Minimum Relative Intensity≥10%. A relative quantification of major, identified compounds was performed on ELSD chromatograms by the peak area integration and expressed as a relative peak area in% of the whole chromatogram.

Bioactivity Assays The standardized Microtox® bioassay (Microbics, Carlsbad, CA, USA) (Marti et al., 2003) was used to assess the bioactivity of *O. tuberculata* crude extract. Crude organic extracts were dissolved in artificial seawater, and up to 2% of acetone was added to improve dissolution of the extracts. Solution was tested in four diluted concentrations. The initial concentration was set at 500 μg/ml, and a dilution factor of 2 was applied between each following tested concentration. Bioactivity was quantified by measuring the direct effect on the metabolism of the bioluminescent bacterium *Vibrio fischeri* indicated by a decrease in light emitted and expressed as an EC50 value.

An antimalarial assay was performed using the *Plasmodium falciparum* FcB1 strain, grown *in vitro* according to Trager and Jensen (Florenta et al., 2000). Parasite culture medium contained RPMI 1640 medium (Life Technologies, Inc.), 25 mM Hepes, 27.5 mM $NaHCO_3$, and 11 mM glucose (pH 7.4), and was supplemented with 7.5% (v/v) compatible heat-inactivated human serum. Human red blood cells (RBC) were added at hematocrit of 2%, and the parasite culture was maintained at 37°C under an atmosphere of 3% CO_2, 6% O_2, and 91% N_2, with daily medium changes. The effect of extracts on the intra-erythrocytic development of *P. falciparum* was performed with cultures containing mixtures of all stages at a final parasitemia of ~1% that then were distributed in 96-well microplates (Trager and Jensen, 1976). Extracts were added to the wells, in parasite culture medium, at final concentrations of 10, 1, and 0.1 μg/ml in duplicates. Parasites were allowed to grow in a candle jar system and after 24 h in culture, ^{3}H-hypoxanthine was added (0.5 μCi/well). After an additional 24 h incubation period, parasites were harvested on filters after a freeze-thawing cycle. Dried filters were submerged in a liquid scintillation mixture (OptiScintHisafe, Perkin Elmer) and counted in a 1450 Microbeta counter (Wallac, Perkin Elmer). Parasite growth inhibition was calculated from the parasite-associated radioactivity (incorporated into nucleic acids) in treated cultures compared with control cultures performed in the presence of equivalent amounts of DMSO but without tested compounds. Each extract was tested in duplicate on a *P. falciparum* strain (FcB1) (Desjardins et al., 1979).

Antitumor assay was performed on KB cell lines that were plated in 96-well tissue culture plates in 200 μl medium, and were treated 24 h later with compounds dissolved in DMSO; compound concentrations ranged from 0.025nM to 6 mM, and were prepared by use of a Biomek 3000 (Beckman). Control cells received the same volume of DMSO (1% final volume). After 72 h exposure to the drug, MTS reagent (Cell titer Aqueous, Promega, Madison, WI, USA) was added and incubated for 3 h at 37°C: the absorbance was monitored at 490 nm, and results are expressed as the inhibition of cell proliferation calculated as the ratio [1 - (OD_{490} treated/OD_{490} control) x 100] (Zampella et al., 2009).

Data Analyses Normality of data distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test) assumptions were satisfied. Therefore, one-way analyses of variance (ANOVA) were used to test the effects of month/season and sponge life stage on the compound's expression levels. Pearson's correlation coefficient was used to assess the correlation between the relative expression level of compounds and the water temperature. Temperature data were provided by the MEDCHANGE project (N. Bensoussan & J. C. Romano). The average weekly and monthly temperatures were calculated by the integration of daily temperatures for 7 and 30 days that preceded the sampling date.

Results

Major Compounds Identifications Two already known 5-alkylpyrrole-2-carbaldehydes were obtained in a mixture

from an apolar fraction of the purification process. Comparison of the ^{1}H NMR and MS data with those of compounds already isolated from the same species enabled us to confirm their structures (Fig. 2).

Two major compounds unknown for Homoscleromorpha sponges also were isolated and characterized from a more polar fraction of the *O. tuberculata* organic extract. Both compounds appeared to be LPLs: the new compound LPE 1, and a lyso-PAF previously reported from a few other sponge species (Fig. 2).

2-O-Docosa-5,11-dienoyl-sn-glycero-3-phosphatidylethanolamine (LPE 1): amorphous solid; ^{1}H NMR (500 MHz, CD_3OD) δ 5.40 (m, 2 H, H-11' and 12'), 5.35 (m, 2 H, H-5' and 6'), 5.00 (quint, ^3J=5.3 Hz, 1 H, H-2), 4.06 (m, 2 H, H-1"), 4.00 (m, 2 H, H-3), 3.70 (m, 2 H, H-1), 3.18 (m, 2 H, H-2"), 2.36 (t, ^3J=7.6 Hz, 2 H, H-2'), 2.10 (q, ^3J= 7.0 Hz, 2 H, H-4'), 2.04 (m, 6 H, H-7', H-10' and H-13'), 1.67 (quint, ^3J=7.6 Hz, 2 H, H-3'), 1.29 (m), 0.90 (t, ^3J= 6.6 Hz, 3 H, H-20'); ^{13}C NMR (125 MHz, CD_3OD) δ 175.0 (C, C-1'), 132.0 (CH, C-12'), 131.1 (CH, C-11'), 130.9 (CH, C-6'), 129.9 (CH, C-5'), 74.6 (CH, C-2), 64.9 (CH_2, C-3), 63.0 (CH_2, H-1"), 61.4 (CH_2, C-1), 41.6 (CH_2, C-2"), 34.7 (CH_2, C-2'), 33.2 (CH_2, C-8' and 9'), 31.0-30.5 (CH_2), 28.3-27.7 (CH_2, C-4', 7', 10' and 13'), 26.2 (CH_2, C-3'), 23.9 (CH_2, C-19'), 14.6 (CH_3, C-20'); ESIMS *m/z* 528.3 $[M+Na]^+$; MS^2(528.3) *m/z* 485.2 $[M–C_2H_4NH_2+Na]^+$, 467.3 $[M–C_2H_4NH_2–H_2O+Na]^+$; MS^3(485.2) *m/z* 387.2 $[M–C_2H_4NH_2–H_2O–HPO_3+Na]^+$; HRESIMS *m/z* 506.32480 $[M+H]^+$ (calcd for $C_{25}H_{49}NO_7P^+$, 506.32466).

LPE 1 was isolated as an amorphous solid contaminated with an inseparable phosphocholine derivative, as evidenced in the ^{1}H NMR spectrum (20% contamination). This impurity made irrelevant the measure of the rotatory power and its biological activity, but the characterization of this new compound nevertheless was undertaken by interpretation of NMR and HRMS data, as well as by GC-MS data after hydrolysis of the fatty acid side-chain. The molecular formula of LPE 1 was established as $C_{25}H_{48}NO_7P$ on the basis of HRESIMS data (*m/z* 506.32480 $[M+H]^+$, Δ +1.35 ppm). An easily identified ^{1}H-^{1}H spin coupling system between δ_H 2.36 (t, ^3J=7.6 Hz, 2 H, H-2'), 1.67 (quint, ^3J=7.6 Hz, 2 H, H-3'), 2.10 (q, ^3J=7.0 Hz, 2 H, H-4'), 5.35 (m, 2 H, H-5' and 6'), 2.04 (m, 2 H, H-7') ppm revealed a $\Delta^{5,6}$ unsaturated acyl chain. A second unsaturation was evidenced with the signals at δ_H 5.40 (m, 2 H, H-11' and 12') in the ^{1}H NMR spectrum. The exact structure of the acyl chain was determined finally after acid catalyzed methanolysis and pyrrolidide derivatization. The position of the second double bond was determined by examination of the GC-EIMS fragmentation pattern. In particular, the pyrrolidide derivative afforded a molecular ion peak at *m/z* 361 $[M]^+$ and a succession of fragments separated by a mass difference of *m/z* 14 except for *m/z* 222 and 234, but also 140 and 152, which placed the unsaturations at C-5' and C-11' of the fatty acid. This acyl chain was placed at the 2-*sn* position of a glycerol core due to the key H-2', H-3', and H-2/C-1' HMBC correlations. The signal at δ_H 5.00 (quint, ^3J=5.3 Hz, 1 H, H-2) ppm, COSY correlated with two signals at δ_H 4.00 (m, 2 H, H-3) and 3.70 (m, 2 H, H-1) ppm, was indeed characteristic of a 1-lysophospholipid. The phosphatidyl part at 3-*sn* was identified finally as a phosphatidylethanolamine due to the COSY correlated signals at δ_H 4.06 (m, 2 H, H-1") and 3.18 (m, 2 H, H-2") ppm. Configurations of the double bonds were assigned as *Z* because of shielded ^{13}C NMR chemical shifts of the allylic carbons C-4', C-7', C-10' and C-13' around 28.0 ppm. Finally, the impurity was strongly suggested to be the previously described lyso-PAF as indicated from similar ^{1}H NMR and MS data.

The known 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (lyso-PAF) was first characterized by ^{1}H NMR analysis, which revealed characteristic COSY correlated signals of an unsaturated *O*-alkyl chain at δ_H 3.47 (t, ^3J=6.6 Hz, 2 H, H-1'), 1.56 (quint, ^3J=6.5 Hz, 2 H, H-2'), 1.29 (m), and 0.90 (t, ^3J=6.6 Hz, 3 H, H-16') ppm. Substitution of this chain at C-1 of the glycerol core was inferred from HMBC interpretation and chemical shifts of H_2-1 and H-2. Another spin correlated system at δ_H 4.42 (m, 2 H, H-1"), 3.70 (m, 2 H, H-2") ppm together with the singlet at 3.23 ppm (9 H, N-$C\underline{H}_3$) suggested the presence of a phosphatidylcholine. Structure of this known compound was confirmed by HRESIMS data interpretation, which were consistent with the molecular formula $C_{24}H_{53}O_6NP$ (*m/z* 482.3610 $[M+H]^+$, Δ +1.14 ppm). After comparison with the literature, all NMR

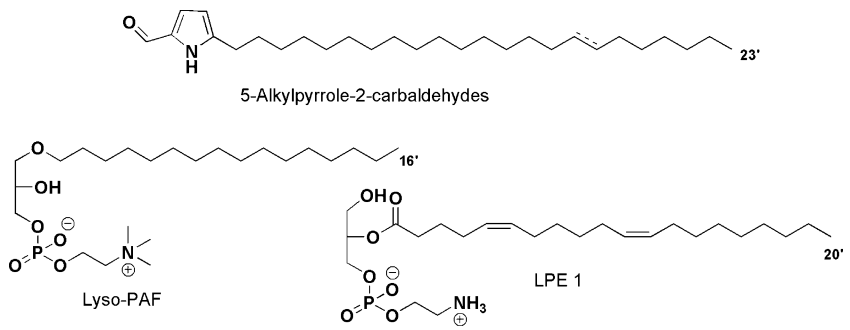


Fig. 2 Chemical structure of the major secondary metabolites isolated from *Oscarella tuberculata*

and MS data were fully in accordance with the known lyso-PAF (Shin et al., 1999).

LPL Temporal Variability LPL expression levels were studied by using metabolic fingerprinting with ELSD detection. Overall, the expression level of LPE 1 was about three times higher than the expression level of lyso-PAF. Both compounds showed significant seasonal fluctuations in their expression levels (Fig. 3), which appeared to be highly correlated (Fig. 4). Over 2 years, maximal values were observed in the summer period, from June to August (4.11% for lyso-PAF and 10.69% for LPE 1 in August 2003). Minimal values were detected from December 2001 to February 2002 (1.68% for lyso-PAF and 3.34% for LPE 1) as well as in September 2002 (1.22% for lyso-PAF and 3.53% for LPE 1). We did not find any significant effect of the natural fluctuations of seawater temperature (average daily, weekly and monthly temperature) on the lyso-PAF or LPE 1 level of expression. However, we observed a progressive increase in LPL production following the warming up of the seawater (from February to August 2002), with the highest expression levels recorded in summer (Fig. 3).

LPL Expression Level in Relation to Sponge Life Cycle Oscarella tuberculata is an ovoviviparous and gonochoric species. It has a seasonal reproductive cycle with gametogenesis starting in January, overlapped and followed by embryogenesis and larval development in summer (from mid-June to early September). Peak reproductive effort was observed during the summer season, with the highest values in June 2002 (8.59%) for males and July 2003 for females (6.79%). For the latter, the maximal reproductive effort thus corresponds to the period of embryogenesis and larval development.

The expression levels of both LPLs varied significantly in relation to the sponge reproductive stage (gametogenesis vs. embryonic and larval development) (Fig. 5). The LPL expression level increased progressively from June, corresponding to the beginning of embryogenesis, and reached the highest values in August during the final stage of larval development (Figs. 3 and 5). The expression level peak was followed by a steep decrease at the end of summer after larval release (Fig. 3). Overall, we observed that LPL expression levels were significantly higher in reproductive sponges than in non-reproductive sponges, and in females rather than in males (Fig. 6).

Crude Extract Bioactivity Oscarella tuberculata crude extract displayed a large panel of bioactivities. An effect on metabolism of the bioluminescent marine bacterium *Vibrio fischeri* was detected at EC_{50}=35.9 µg.ml^{-1} (standardized Microtox® bioassay). A moderate antitumoral activity of *O. tuberculata* crude extract against human cancer KB cell line was also observed at 10 µg.mL^{-1} (96.5% of cytotoxicity). Finally, the same extract exhibited antimalarial activity against the *Plasmodium falciparum* (FcB1 strain) at the same concentration (38.9%).

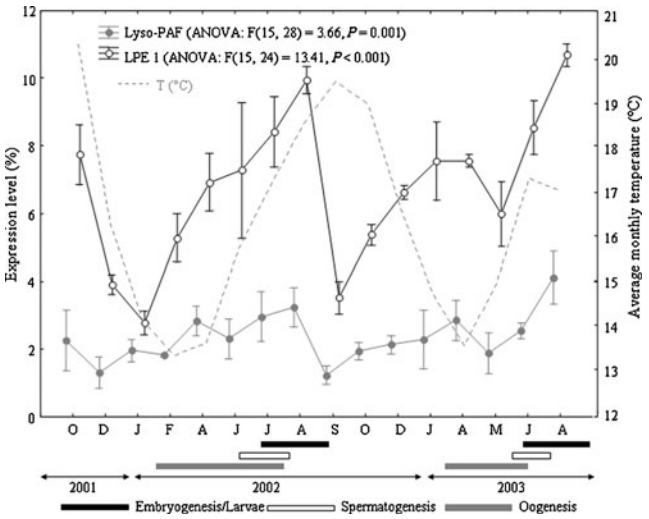


Fig. 3 Temporal variability in lyso-PAF and LPE 1 expression level (%), from October 2001 to August 2003, in parallel to seasonal fluctuations of seawater temperature. The expression level of each compound is calculated according to peak area, and expressed as a percentage of the whole chromatogram. Different stages of the reproductive cycle (oogenesis, spermatogenesis, and embryogenesis) are indicated on the temporal scale

Discussion

Although PL and LPL class composition and their relative abundance in different sponge species have been studied

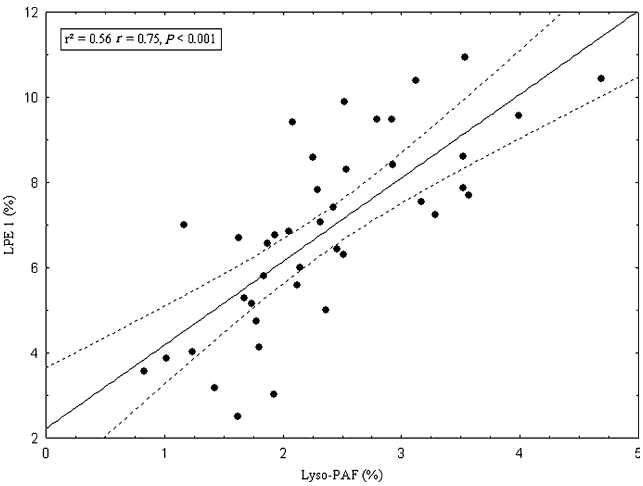


Fig. 4 Correlation between expression levels of lyso-PAF and LPE 1. The expression level of each compound is calculated according to peak area, and expressed as a percentage of the whole chromatogram

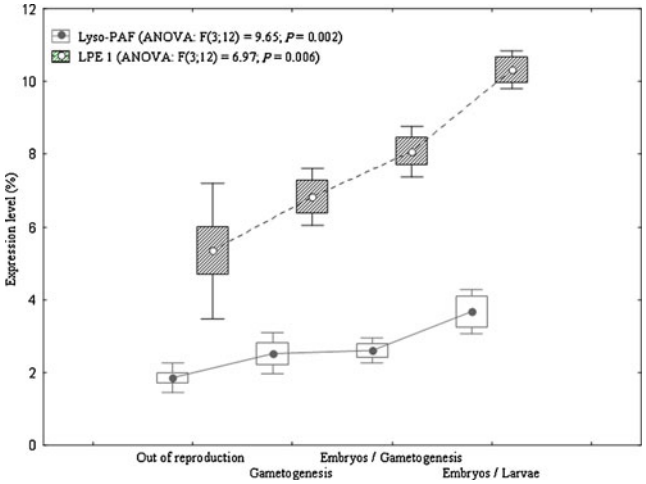


Fig. 5 Variation of lysophospholipid contents according to the different periods of the sponge life cycle. The expression level of each compound is calculated according to peak area and expressed as a percentage of the whole chromatogram

previously (Dembitsky et al., 1989; Genin et al., 2008), only a few chemical studies have reported the isolation and structural elucidation of LPLs in this taxonomic group (Butler et al., 1996; Shin et al., 1999; Alam et al., 2001; Lee et al., 2003; Zhao et al., 2003; Müller et al., 2004; Mansoor et al., 2005). We isolated and identified two LPLs that were unknown for *O. tuberculata*, a lyso-PAF C16, previously reported in a few other sponge species (Table 1) and a new lysophosphatidylethanolamine C20:2 (LPE 1). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) seem to be two major PL classes in sponges together with their LPL counterparts (Dembitsky et al., 1989; Genin et al., 2008). The new LPE is original because of the presence of C20:2 fatty acid with unsaturations Δ5,11. The occurrence

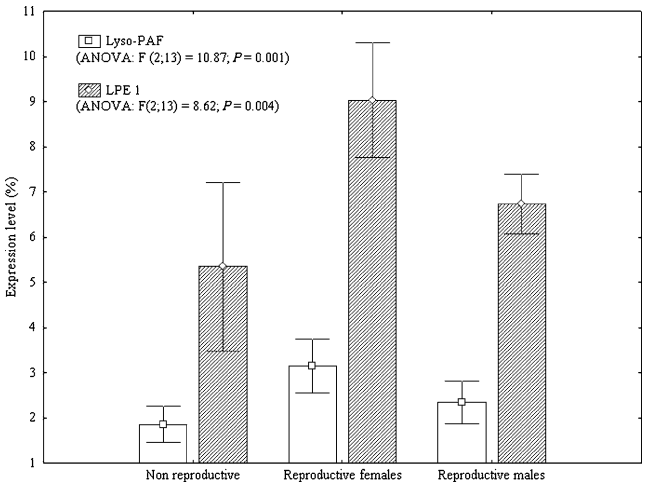


Fig. 6 Mean expression levels of lysophospholipids according to sponge sex. The expression level of each compound is calculated according to peak area, and expressed as a percentage of the whole chromatogram

of non-methylene-interruped fatty acids and especially 20:2 Δ5,11 has been reported previously from marine invertebrates, and mostly from mollusks, but they are less frequent in marine sponges (Barnathan, 2009).

The discovery of potent biological activities associated with LPLs has stimulated the research on their metabolic functions and potential physiological roles. Bioactivities of some sponge LPLs have been investigated by using various *in vivo* and *in vitro* bioassays. For example, the sponge *Suberites domuncula* showed an adaptive antibacterial reaction, which appeared to be linked to an increase in the level of lyso-PAF derivatives in response to endotoxins production (Müller et al., 2004). A lyso-PAF isolated from *Crella incrustans* was identified as an active antifouling agent by using a series of bioassays (Butler et al., 1996). Its activity was suggested to be related to the LPL amphiphilic nature and its surfactant properties that can prevent biofilm formation. Additionally, lyso-PAF analogues isolated from *Spirastrella abata* and *Stelletta* sp. have shown cytotoxicity against human tumor cell lines and an inhibitory effect on cholesterol biosynthesis (Shin et al., 1999; Alam et al., 2001; Zhao et al., 2003). The lyso-PAF structural features also underline their ability to interfere with ion transport mechanisms, thus exerting an effect on channel proteins and/or membranes that are involved in intracellular communication processes. *Oscarella tuberculata* crude extract showed the highest bioactivity among Homoscleromorph species as detected by the standardized Microtox test. Another well-studied Homoscleromorph, *Corticium candelabrum,* which produces cytotoxic and antibacterial aminosterols (plakinamines), had a bioactivity about five times lower than *O. tuberculata* (Ivanišević et al., 2011). *Oscarella tuberculata* antitumoral and antimalarial activities also are comparable to that of *C. candelabrum* (unpublished data). These biological assays, of course, do not relate directly to the ecological function(s) of the compounds, but rather provide insight into their pharmacological potential. However, their antibacterial and cytotoxic activity may be a good indication of the sponge's ability to prevent biofilm formation during the fouling process.

Regarding the displayed activity of *O. tuberculata* crude extract and the activity reported for lyso-PAF analogues in other sponges, the isolated lyso-PAF from *O. tuberculata* may indicate an allelochemical function as a fouling deterrent. Such a chemical defense is particularly important in benthic hard substrate communities where sessile organisms are subjected to fouling and competition for space and resources (Becerro et al., 1997). The hypothesis is supported by our in situ observations; indeed *O. tuberculata* is never overgrown by other invertebrate species, and it is often observed as an epibiont on other massive sponges or erected cnidarians.

LPL expression levels showed a strong seasonality, with a minimum in winter and a maximum in summer.

Variations in expression levels of both LPLs appeared to be highly correlated, which suggests that their production is probably related and/or dependent on the same metabolic pathways. Similar seasonality in the production of secondary metabolites has been observed previously in marine invertebrate species (Turon et al., 1996; Page et al., 2005; Abdo et al., 2007). Variability in metabolite expression levels may be linked to the fluctuation of environmental parameters such as temperature and/or nutrient bioavailability. We noticed that the highest LPL expression levels were recorded in summer, although no correlation was found between variations in LPL expression levels and natural fluctuations of seawater temperatures. However, moderate elevated temperature could have a stimulating effect on metabolite biosynthesis. We also assume that biotic factors and especially the physiological state of the organism should have a significant higher effect on secondary metabolite production. Indeed, seasonal variability of secondary metabolism may result from a trade-off with primary biological functions such as reproduction. LPL variability, with peak expression during the period of embryogenesis and larval development is noteworthy, suggesting a physiological function rather than an ecological role. It is known that some LPLs act in a wide range of animal cells as growth factors with hormone-like biological activities. They initiate and regulate cell proliferation including mitosis, cell differentiation and migration, and affect cell viability through anti-apoptosis properties (Birgbauer and Chun, 2006; Torkhovskaya et al., 2007). Considering their role among metazoans, one hypothesis is that they act as signaling molecules or metabolic intermediates during embryogenesis. The strong decreased concentration in sponges with released offspring is another clue to their putative role in larval development. Additionally, a secondary role as precursors of *de novo* synthesized glycerolipids used as energetic reserves cannot be excluded.

To summarize, we identified two lysophospholipids that are major and novel molecules for *O. tuberculata*. The isolation of sponge lysophospholipids has been rarely reported. Rather than a lack of interest, this probably has been due to purification difficulties. The recording of potent bioactivities justifies further research on their biological functions. In this regard, we have provided baseline expression levels of two major LPLs from *O. tuberculata,* and put forward a hypothesis concerning their putative biological function. The results suggest that they may have a key role in sponge embryogenesis and morphogenesis, either as signaling molecules that regulate cell division and differentiation, or simply as lipid reserves. Considering the high energy costs of chemical defenses, an optimized allocation of resources should be to produce secondary metabolites with multiple functions (Hartmann, 2007). Regarding the LPL amphiphilic character and the reported antimicrobial and cytotoxic activities, *O. tuberculata* LPL also might play an additional role as antifouling agents to prevent overgrowing or predation by other dwellers of hard substrate communities. In order to confirm these hypotheses, further research is necessary.

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OBITUARY

For Love of Eisner

Thomas Eisner (1929–2011)

Robert A. Raguso

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On March 25, 2011, Chemical Ecology lost one of its original guiding lights, Thomas Eisner, after a long and courageous battle with Parkinson's Disease. By now, numerous obituaries have detailed his prolific career as a research scientist, photographer, musician and champion of environmental and human rights. Equally well documented is his intriguing personal odyssey, beginning as a child refugee from fascist Europe and ending as a beloved professor emeritus at Cornell University. Others have profiled his numerous awards in recognition of his excellence in research (National Medal of Science, Carty Award of the National Academy of Sciences), his ability to communicate the joy of scientific discovery (Lewis Thomas Prize, New York Film Festival Grand Prize) and his tireless dedication to conservation (Tyler Prize for Environmental Achievement). Instead, in this brief essay I celebrate Tom's unusual scientific vision, rooted in the synergism between natural history and the experimental study of mechanism, and nurtured through collaborations across the physical sciences. The Eisner Vision figured prominently in the establishment of Chemical Ecology as a field, as well as the founding of my home department (Neurobiology and Behavior) at Cornell. It is carried forward today by his former students and remains a unifying philosophy among the new generation of chemical ecologists at Cornell, with whom I have the privilege of working.

Tom Eisner's keen interest in natural history was kindled during his childhood in Uruguay, described glowingly in his autobiographical "For Love of Insects". Aside from Niko Tinbergen's "Curious Naturalists", I find this book unrivaled in its humble and joyous account of a life spent pondering nature. Its passages resonate with anyone whose scientific career was sparked by early encounters with the natural world and was sustained by a desire to understand its inner workings. As an adult, Tom continued to seek inspiration through critical observations of natural history, accompanied by his wife and lifelong collaborator Maria (Loebell) Eisner, the naturalist Mark Deyrup, and their students. These observations invariably were followed by bioassays in which hapless frogs, birds, spiders, ants or fish were duped into approaching a would-be prey item, only to get splattered with some noxious defensive secretion. Although Tom's explorations required increasingly more sophisticated tools, these only enhanced, rather than diminished, the Eisnerian sense of wonder so familiar to generations of Cornell students.

Tom was a great aficionado of analytical equipment, especially anything that pushed the limits of optics, microscopy and high speed/resolution photography. So many of his group's seminal contributions, including the mechanisms of benzoquinone emission by bombardier beetles, the adhesion of the palmetto beetle's tarsal bristles to leaf surfaces, and the "hidden" patterns of UV reflectance in flower petals, were communicated through stunning photographic images. As accomplished a photographer as Tom was, he benefited from (and lovingly acknowledged) Maria's mastery of SEM, and frequently recounted in lectures his awestruck encounter with Harold "Doc" Edgerton, MIT's pioneer of strobe photography, whose high speed wizardry revealed the cooling mechanism for the abdominal emission chambers of bombardier beetles. Tom also enjoyed a long and fruitful collaboration with Dan Aneshansley, an engineer who devised elegant methods for measuring the physical properties (e.g. heat evolution, force transduction) by which beetles defend

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themselves against predators. Of course, it was Tom's career-long partnership with Jerry Meinwald, whose group provided critical expertise in analytical chemistry, which had the greatest impact on our field. Tom and Jerry's decades of identifying strange compounds from even stranger glands led them through a forest of natural products, from the C10 cyclopentanoid constituents of catnip (nepetalactone) and walking stick defensive sprays (anisomorphal), to carminic acid, lucibufagin steroids and pyrrolizidine alkaloids that protect cochineal bugs, fireflies and tiger moths, respectively, from a grisly death. In the quality and quantity of their collaborative output, Eisner and Meinwald were the "Lennon and McCartney" of Chemical Ecology, and their "greatest hits" continue to inspire students of chemical defense, counter-defense and mimicry. As interest (and expertise) in natural product elucidation continues to wane among university chemistry departments, we are reminded of how crucial such collaborations are to the continued growth of our field. During his last years, this remained one of Tom's greatest concerns.

Tom sometimes lamented that his research had been criticized for its "lack of conceptual framework", but this criticism was shortsighted. In his foreword to "For Love of Insects", Tom's friend and intellectual gadfly E.O. Wilson likened him to a pointillist painter, from whose body of focused, detailed case studies emerges a canvas rich with patterns "of evolutionary adaptation, molecular evolution, behavior and life cycles that likely would not have been revealed by other means". As a group, chemical ecologists have been slow to grasp evolutionary theory and slower to embrace conditionality in the interactions whose mechanisms they dissect with such care. Not so with Tom and his students, whose studies of butterfly courtship, tiger moth alkaloid acquisition, spider web construction or herbivore-induced nicotine mobilization were rife with fitness tradeoffs, cost-benefit analyses and contingency. The students responsible for these studies are among today's most conceptually sophisticated interpreters of animal (and plant) behavior. Like Tom, their current research builds upon the foundation of natural history with sophisticated experimental manipulations of visual and acoustic as well as chemical and metabolic signals, in studies designed to explicitly measure fitness consequences.

Finally, Tom felt that by combining aesthetically appealing patterns with rigorous experimental elucidation of their underlying processes, he could more effectively communicate the splendor and importance of the natural world to a broader public, and educate them on the dire need to conserve nature's pharmacopoeia for future generations. In the final passage of "For Love of Insects", Tom described the mutual dependence between the role of nature in sustaining human curiosity and the increasingly urgent role of human curiosity (and its valuation) in preserving the world's remaining wild places. "Will the collective urge to discover keep natural history alive?" he asked. "Without [human] curiosity, without a passion for discovery, nature cannot endure. And without nature, curiosity will fade…It is so fundamentally human to thirst for knowledge and to turn to nature for visions of the unknown".

Thomas Eisner inspired generations of chemical ecologists to turn to nature for chemical visions of the unknown, visions that have given form and structure to the increasingly complex interaction webs that we now study in terrestrial, aquatic and marine environments across the globe, as communicated in this journal. He will be sorely missed by those who were inspired by his vision and his unquenchable sense of wonder about the natural world. I will remember him fondly, along with his tales of toxic steroids and *femmes fatales*, whenever fireflies light up the humid summer evenings of Ithaca.

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RAPID COMMUNICATION

Near-Infrared Imaging Spectroscopy as a Tool to Discriminate Two Cryptic *Tetramorium* Ant Species

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Abstract Correct species identification is a precondition for many ecological studies. Morphologically highly similar, i.e., cryptic, species are an important component of biodiversity but particularly difficult to discriminate and therefore understudied ecologically. To find new methods for their rapid identification, thus, is important. The cuticle's chemical signature of insects often is unique for species. Near-infrared spectroscopy (NIRS) can capture such signatures. Imaging NIRS facilitates precise positioning of the measurement area on biological objects and high-resolution spatial capturing. Here, we tested the applicability of imaging NIRS to the discrimination of cryptic species by using the ants *Tetramorium caespitum* and *T. impurum*. The classification success of Partial Least Squares Regression was 98.8%. Principal Component Analysis grouped spectra of some *T. impurum* individuals with *T. caespitum*. Combined with molecular-genetic and morphological evidence, this result enabled us to pose testable hypotheses about the biology of these species. We conclude that discrimination of *T. caespitum* and *T. impurum* with imaging NIRS is possible, promising that imaging NIRS could become a time- and cost-efficient tool for the reliable discrimination of cryptic species. This and the direct facilitation of potential biological insight beyond species identification underscore the value of imaging NIRS to ecology.

Key Words Cuticular hydrocarbons · Ant worker · Infrared spectroscopy · Chemical fingerprinting

Introduction

Species are basic units of ecology. However, species identification can be difficult, especially with morphologically nearly identical, i.e., cryptic, species. This situation hampers the ecological study of cryptic species, now known from many groups of organisms.

Near-infrared spectroscopy (NIRS) can afford an opportunity to make cryptic species accessible to ecology. By using the characteristic reaction of functional groups to infrared light in order to assign peaks to these functional groups, a fast and 'easy-to-use' identification tool has resulted (Workman and Weyer, 2008). It is increasingly used in many disciplines, including in entomology, as the cuticle's chemical signature is important for species discrimination in insects (Steiner et al., 2002; Martin and Drijfhout, 2009; Sikulu et al., 2010; Seppä et al., 2011). However, an exact positioning can be difficult for small specimens. Imaging-NIRS technology allows precise positioning of the measurement area and spectra capturing at a high spatial resolution (up to 6.25×6.25 μm), and thus has potential advantages over classical, fiber-optic-probe NIRS, where each spectrum represents an area of several square millimeters.

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Imaging NIRS has not been used for cryptic-species discrimination so far. Here, we investigated whether the cryptic ant species *Tetramorium caespitum* and *T. impurum*, which otherwise can be identified only via molecular genetics or high-precision morphometrics (Schlick-Steiner et al., 2006), can be discriminated by their NIR spectra.

Methods and Materials

We selected 15 nest samples for each species from various European countries, collected in –2007 and already species-identified using mitochondrial (mt) DNA and morphometrics (Schlick-Steiner et al., 2006; Steiner et al., 2010). Individuals had been killed with and stored in 99% ethanol p.a. A plane measurement area that could be identically positioned in all individuals was chosen, i.e., a 400×400 μm frons area. Thus, the heads of three workers per nest were stuck on a microscope slide with wallpaper paste in such a way that head width and length were maximum. We excluded incompletely colored, i.e., very young workers.

Spectra were recorded using a Spectrum 400 (Perkin Elmer) spectrometer and a Spotlight 400 microscope with a nitrogen-cooled MCT detector array (16×2). At a resolution of 25×25 μm, we collected 256 spectra per individual from the 400×400 μm area, for wavelengths 7,800–2,000 cm^{-1}. The 256 spectra were reduced to one spectrum representing the measurement area. Data were analyzed using The Unscrambler 10.0.1 (Camo).

Spectra obtained from a smaller region (150×150 μm) for the individuals of nests TM45, TM308, i62, TM235, TM290, and i36 were used in a pre-test (data unpublished). As spectra of the TM45 and TM308 individuals could not be separated from *T. caespitum* spectra by Principal Component Analysis (PCA; see results), DNA was extracted from these six decapitated individuals, and a cytochrome-C-oxidase subunit one fragment of mtDNA was reverse sequenced as in Schlick-Steiner et al. (2006), with slight modifications.

For statistical analysis, an unsupervised method, PCA, and a supervised method, Partial Least Squares (PLS) Regression (NIPALS algorithm) were used. We randomly chose 14 *T. caespitum* and 15 *T. impurum* spectra as a validation set. For PCA, data were mean-centred; the first and second PC were plotted. For PLS Regression, data were mean-centred and converted to first Savitzky-Golay derivative. We set the constituent to 1 and 2 for *T. caespitum* and *T. impurum* individuals, respectively. Calibration quality was evaluated using R^2, root mean standard error of calibration (RMSEC), root mean standard error of prediction (RMSEP), and validation BIAS.

Regression coefficient plots were noisier in regions 7,800–5,500 and 2,500–2,000 cm^{-1}; therefore, we compared the regression coefficient plots of region 5,500–2,500 cm^{-1}.

Results

Heads of four individuals were destroyed during the measurement procedure. After checking spectra manually

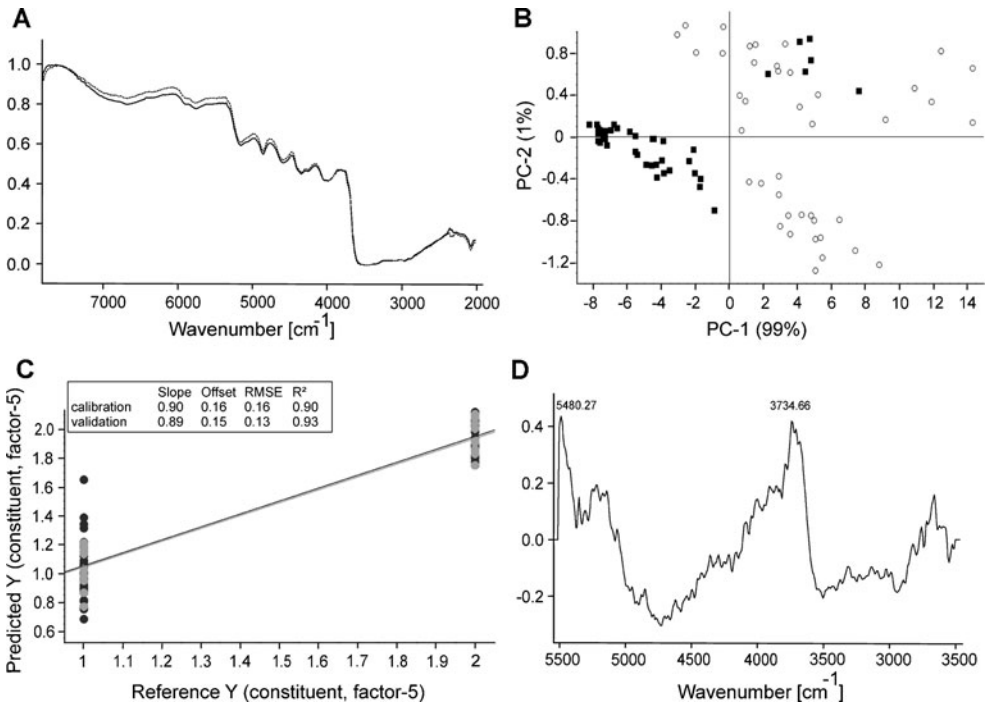


Fig. 1 Differences between species. **a** Example mean spectra (normalized 0–1) of a *Tetramorium caespitum* (*dotted line*) and a *T. impurum* (*solid line*) individual. **b** PCA of 84 individuals' mean spectra. Circle: *T. caespitum*; square: *T. impurum*; the six squares in the range of *T. caespitum* represent the individuals of *T. impurum* nests TM45 and TM308. **c** PLS Regression, predicted vs. reference plot using five factors of 84 individuals for the calibration set (*black*) and 29 individuals for the validation set (*grey*). **d** Regression coefficient plot of region 5,500–2,500 cm^{-1} for five factors

and via PCA, two outlier mean spectra were excluded. The remaining 84 spectra (for examples, see Fig. 1a) were used for the analyses.

In the PCA, the two species were separated, except for two nests (TM45, TM308; Fig. 1b) genetically and morphologically assigned to *T. impurum* by Schlick-Steiner et al. (2006). Our mtDNA analysis here of exactly those TM45 and TM308 individuals analyzed by NIRS also resulted in *T. impurum*. However, the independent imaging-NIRS pre-test had led to the same discrimination problem in PCA (data unpublished). When not considering TM45 and TM308, *Tetramorium impurum* showed less variability in its spectra than *T. caespitum*.

The PLS calibration was performed using five factors, and resulted in a correct classification of 98.8%, an R^2 of 0.90 for calibration and of 0.93 for validation. RMSEC and RMSEP were 0.16 and 0.13, respectively, and validation BIAS was −0.01, meaning that the spectra used for calibration supported the separation and prediction of the two species (Fig. 1c).

The regression coefficients had two distinct peaks in the five-factor plot at 3,730 and 5,481 cm^{-1} (Fig. 1d).

Discussion

Our results suggest that cryptic ant species can be separated by their near-infrared spectra. The two distinct peaks in the regression-coefficient plots correspond to hydrocarbon overtone vibration (Workman and Weyer, 2008), suggesting that these peaks represent species-diagnostic characters.

Concerning the two *T. impurum* nests not assigned to this species via PCA, we exclude sample confusion and misidentification, because the six scanned individuals were assigned to *T. impurum* by mtDNA, and spectra of these individuals also clustered with *T. caespitum* in the NIRS pre-test. An influence of the preservation method over time (cf. Perez-Mendoza et al., 2002) is also unlikely to be causal to the situation because samples did not group according to age in the PCA, and other samples collected in the same years as TM45 (2000) and TM308 (2002) were discriminated correctly. Instead, we are aware of three possible biological explanations, now available for future testing: (*i*) Interspecific hybridization was inferred for the *T. caespitum/impurum* complex (Steiner et al., 2010). Mitochondria are passed uniparentally rendering hybridization undetectable by mtDNA alone. If, for example, TM45 and TM308 were older hybrids, i.e., not F1 hybrids, different identification results from morphometrics and NIRS (*T. caespitum*) could reflect different selection pressures on the morphological and the chemical phenotype. (*ii*) We had excluded very young workers, but the age of the included individuals could still influence CHC profiles as known from other insects (Sikulu et al., 2010). (*iii*) TM45 and TM308 could have rare CHC profiles, and our *T. impurum* sample might under-represent the true variability of that species. Possibly in line with this, *T. impurum* was less variable in its spectra than *T. caespitum* when the two 'problematic' nests were excluded. However, two *Formica* ant species also differed strongly in CHC variability (Seppä et al., 2011), and such pattern could, alternatively, be common for closely related ant species.

With imaging NIRS, hands-on time for sample preparation and measurement was 25 min per individual, at 0.01 Euro costs. Imaging NIRS, thus, is faster than COI sequencing and morphometrics (85 and 75 min, respectively) and cheaper than COI sequencing (10.20 Euro); morphometrics causes no costs. Fiber-optic NIRS is even faster (Sikulu et al., 2010) and, like morphometrics, incurs no costs, but our imaging-NIRS-based PLS-classification success was higher when compared to that in fiber-optic-probe NIRS studies (e.g., Jia et al., 2007; Sikulu et al., 2010). Not included in the scanning area, the surface a specimen is placed on cannot influence measurements with imaging NIRS, and this might be one reason for the particularly good discrimination result. Biological insight beyond species identification may be facilitated by capitalizing on the incongruence for two nests across the results of the NIRS-based PCA and mtDNA- and morphometrics-based species identification. Finally, by allowing exact positioning of the measurement area and image capturing at a high resolution, investigating whether the chemical signature differs across body parts will be possible. This could open up additional possibilities for, e.g., NIRS-based behavioral-ecological studies (e.g., Newey et al., 2008). Thus, imaging NIRS gives a time- and cost-efficient opportunity to make cryptic species accessible to ecology and to further investigate their ecological significance.

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Di- and Tri-fluorinated Analogs of Methyl Eugenol: Attraction to and Metabolism in the Oriental Fruit Fly, *Bactrocera Dorsalis* (Hendel)

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Abstract Oriental fruit fly, *Bactrocera dorsalis* (Hendel), males are attracted to the natural phenylpropanoid methyl eugenol (ME). They feed compulsively on ME and metabolize it to ring and side-chain hydroxylated compounds that have both pheromonal and allomonal properties. Previously, we demonstrated that mono-fluorination at the terminal carbon of the ME side-chain significantly reduced metabolic side-chain hydroxylation, while mono-fluorination of ME at position 4 of the aromatic ring blocked ring-hydroxylation but surprisingly enhanced side-chain hydroxylation. Here, we demonstrated that the introduction of fluorine atoms on both the ring and side-chain of ME blocks both positions that undergo enzymatic hydroxylation and, in particular, completely inhibits oxidative biotransformation of the allyl group. In laboratory experiments, *B. dorsalis* males initially were more attracted to both 1-fluoro-4,5-dimethoxy-2-(3,3-difluoro-2-propenyl)benzene (**I**) and 1-fluoro-4,5-dimethoxy-2-(3-fluoro-2-propenyl)benzene (**II**) than to ME. However, both **I** and **II** were taken up by flies at rates significantly less than that of ME. Flies fed with difluoroanalog **II** partially metabolized it to 5-fluoro-4-(3-fluoroprop-2-en-1-yl)-2-methoxyphenol (**III**), and flies fed with trifluoroanalog **I** produced 4-(3,3-difluoroprop-2-en-1-yl)-5-fluoro-2-methoxyphenol (**V**), but the rates of metabolism relative to rates of intakes were much lower compared to those of ME. Flies that consumed either the tri- or difluorinated analog showed higher post-feeding mortality than those that fed on methyl eugenol. In field trials, trifluoroanalog **I** was ~90% less attractive to male *B. dorsalis* than ME, while difluoroanalog **II** was ~50% less attractive. These results suggest that increasing fluorination can contribute to fly mortality, but the trade off with attractancy makes it unlikely that either a di or trifluorinated ME would be an improvement over ME for detection and/or eradication of this species.

Key Words Oriental fruit fly · *Bactrocera dorsalis* (Hendel) · 1-(3,3-Difluoroprop-2-en-1-yl)-2-fluoro-4,5-dimethoxybenzene · 1-Fluoro-2-(3-fluoroprop-2-en-1-yl)-4,5-dimethoxybenzene · Methyl eugenol · Metabolism inhibition · Field attraction · Bioassay · Male annihilation

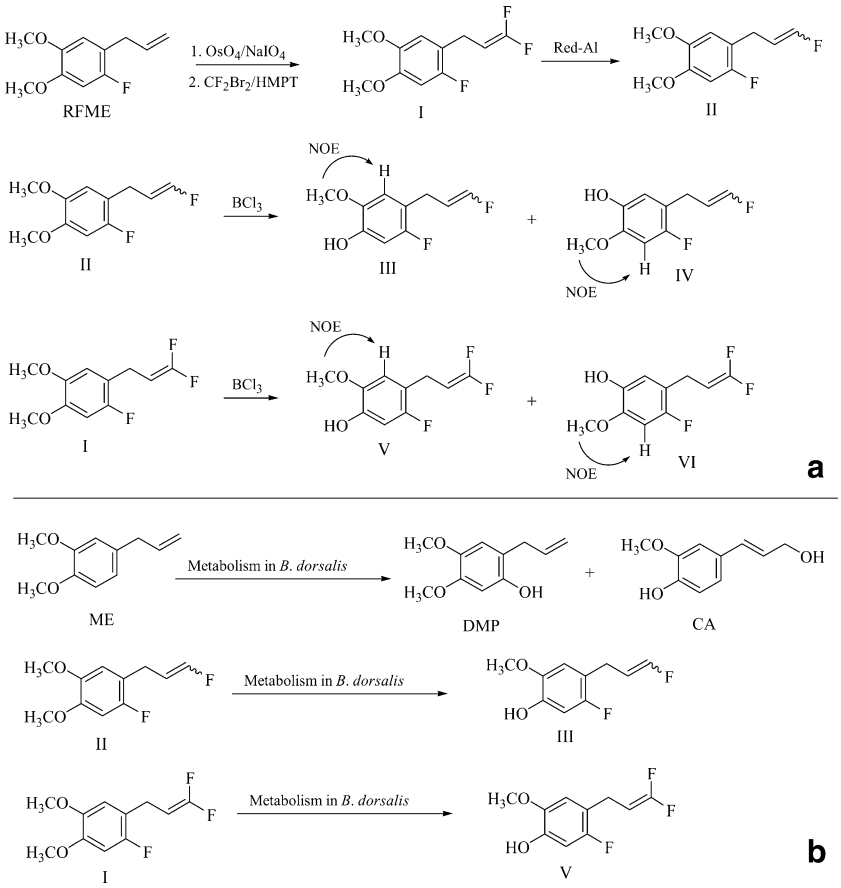
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Introduction

The Oriental fruit fly, *Bactrocera dorsalis* (Hendel), is a serious pest of crops of tropical agriculture, attacking over 173 varieties of fruits and vegetables (Metcalf and Metcalf, 1992). *Bactrocera dorsalis* males are strongly attracted to, and compulsively feed on, methyl eugenol (ME, Scheme 1; Steiner, 1952), a commonly occurring plant phenylpropanoid (Metcalf, 1990). The use of ME in traps and killing



Scheme 1 **a** Syntheses of difluoroanalog **II** and trifluoroanalog **I**, as well as metabolites **III** and **V**. **b** Metabolism of methyl eugenol (ME), difluoroanalog **II**, and trifluoroanalog **I** in *Bactrocera dorsalis*

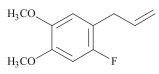
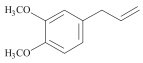
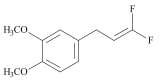
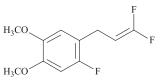
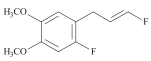
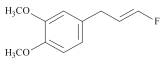
stations is an integral part of successful detection, control, and eradication strategies for *B. dorsalis* populations worldwide (Steiner et al., 1965; Koyama et al., 1984). The attraction to, and consumption of, ME by *B. dorsalis* and sibling species of the *dorsalis* complex, *B. papayae* and *B. carambolae*, also have ecological significance in the communication system of the fly. Mature males, after feeding on ME, metabolize it primarily to 2-(2-propenyl)-4,5-dimethoxyphenol (DMP) and (*E*)-coniferyl alcohol (CA), which exhibit pheromonal and allomonal properties (Nishida et al., 1988; Tan and Nishida, 1996).

One of the potential problems with the continued use of ME for fruit fly control is its reported carcinogenicity (Sekizawa and Shibamoto, 1982; Miller et al., 1983; Schiestl et al., 1989). Metabolic activation of ME and production of 1′-hydroxy metabolites through enzymatic side-chain hydroxylation are thought to be primarily responsible for hepatotoxicity and carcinogenicity of this compound (Smith et al., 2002). The search for safer alternative attractants has led to the evaluation of a number of phenylpropanoids with structural similarities to ME (Metcalf et al., 1975; Mitchell et al., 1985; Khrimian et al., 1993, 1994, 2006, 2009; Liquido et al., 1998).

Two such analogs of ME are 4-[(2*E*)-3-fluoroprop-2-en-1-yl]-1,2-dimethoxybenzene (FME, Table 1), an analog fluorinated at the terminal carbon of the ME side chain, and 1-fluoro-4,5-dimethoxy-2-(prop-2-en-1-yl)benzene (RFME, Scheme 1), an analog fluorinated at the 4 position of the ME aromatic ring. In field tests, FME was as attractive to *B. dorsalis* males as ME (Khrimian et al., 1994), while RFME was ~50% less attractive than ME (Khrimian et al., 2009). In tests, the release rates of RFME and ME were not different (Khrimian et al., 2009), whereas FME was more persistent than ME and, remarkably, about twice as active behaviorally as the corresponding *Z* isomer (Khrimian et al., 1994; Liquido et al., 1998). In laboratory experiments, both FME and RFME attracted, and were readily consumed by, *B. dorsalis* males at rates similar to that of ME (Khrimian et al., 2006, 2009). Interestingly, the degree of biotransformation inhibition is different between the fluorinated analogs, with RFME being completely blocked to enzymatic hydroxylation to DMP (Khrimian et al., 2009), whereas FME is only partially blocked to enzymatic hydroxylation to fluorinated CA (Khrimian et al., 2006). This is probably because FME still has a potential hydroxylation site, following isomerization, at the terminal double-bond position. Overall metabolism by flies also was differentially affected, with RFME enhancing the overall rate while FME did not.

The inhibition of ME biotransformation pathways of FME and RFME is particularly interesting in light of *B.*

Table 1 Residual analysis (compound half-life ± SE) of cotton wicks impregnated with different methyl eugenol analogs and exposed under field conditions. Letters represent significant differences ($P<0.05$) among the half-lives (ANOVA, followed by SNK test)

Name	1-Fluoro-4,5-dimethoxy-2-(prop-2-en-1-yl)benzene	1,2-Dimethoxy-4-(prop-2-en-1-yl) benzene	4-(3,3-Difluoroprop-2-en-1-yl)-1,2-dimethoxybenzene	1-(3,3-Difluoroprop-2-en-1-yl)-2-fluoro-4,5-dimethoxybenzene	1-Fluoro-2-(3-fluoroprop-2-en-1-yl)-4,5-dimethoxybenzene	4-[(2*E*)-3-Fluoroprop-2-en-1-yl]-1,2-dimethoxybenzene
Abbreviation	RFME	ME	DFME	**I**	**II**	FME
Structure						
Half-life ± SE (days)	0.59 ± 0.01a	0.97 ± 0.04ab	1.2 ± 0.1b	1.4 ± 0.3b	2.14 ± 0.07c	3.27 ± 0.07d

dorsalis mortality caused by ME ingested in large amounts (Steiner, 1952). Additionally, the discovery that Melon fly males are attracted to wild orchids (*Bulbophyllum* sp.), which contain ME and in some instances CA and DMP (Tan et al., 2002, 2006), and utilize the metabolized byproducts as pheromonal semiochemicals (Tan and Nishida, 1996), further piqued our interest. In particular, we were interested as to whether or not blocking the metabolic pathway could lead to a single molecule that would be both attractive and more toxic, thus dispensing with the need for additional toxicants. While consumption of neither FME nor RFME increased mortality in *B. dorsalis* males, we were interested to observe whether ME analogs fluorinated at both hydroxylation sites would cause mortality through higher concentrations of unmetabolized phenylpropanoid.

Here, we present the results of our studies on behavioral bioassays and metabolism of two additional fluorinated ME analogs, 1-(3,3-difluoroprop-2-en-1-yl)-2-fluoro-4,5-dimethoxybenzene (**I**, Scheme 1), a ME analog trifluorinated at the 4 position of the aromatic ring and at the terminal carbon of the side chain, and 1-fluoro-2-(3-fluoroprop-2-en-1-yl)-4,5-dimethoxybenzene (**II**, Scheme 1), a ring and side-chain difluorinated analog. Of particular interest is how fluorination of both key metabolic hydroxylation sites of ME affects the overall biotransformation in *B. dorsalis*. Thus, we quantified the attractiveness of compounds **I** and **II**, compared to ME and the previously synthesized analogs FME, RFME, and 4-(3,3-difluoroprop-2-en-1-yl)-1,2-dimethoxybenzene (DFME, Table 1).

Methods and Materials

Analytical Procedures ^{1}H NMR spectra were obtained either at 400 MHz on a Bruker AV-400, or at 500 MHz on a Bruker DRX-500, using TMS as an internal standard. Chemical shifts are reported in δ units, and coupling constants in Hz. Electron-ionization (EI) mass spectra were obtained at 70 eV with an Agilent Technologies 5973 mass selective detector (MS) interfaced with a 6890 N gas chromatograph (GC) equipped with a 30 m×0.25 mm i.d. x 0.25 μm film HP-5MS column. Column temperature was held at 100°C for 2 min and then raised to 260°C at 10°C. min^{-1}. Helium was used as a carrier gas at a constant flow of 1 ml.min^1. GC analyses were performed on a Shimadzu 17A (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) equipped with a flame ionization detector, an auto sampler (AOC-20s) and auto injector (AOC-20i), and an HP-5 capillary column (30 m×0.25 mm×0.25 μm film). Hydrogen was used as carrier gas at 1 ml.min^1. Column temperature was maintained at 100°C for 2 min and then raised to 260°C at 10°C.min^1. Thin layer chromatography (TLC) analyses were conducted using Whatman AL SIL G/UV plates, with hexanes/ethyl acetate as a mobile phase and a 20% solution of phosphomolybdic acid in ethanol and/or UV for visualization of spots. Flash chromatography was carried out with 230–400 mesh silica gel (Fisher Scientific, Fair Lawn, NJ, USA).

Chemicals All reagents and solvents were purchased from Aldrich Chemical Co. unless otherwise specified. Dichloromethane (DCM) was distilled from calcium hydride, and tetrahydrofuran (THF) was dried by distillation from sodium benzophenone-ketyl. Methyl eugenol metabolites, 4,5-dimethoxy-2-(2-propenyl)phenol (DMP) and (*E*)-coniferyl alcohol (CA) were prepared from ferulic acid (Quideau and Ralph, 1992) and 3,4-dimethoxyphenol (Benbow and Katoch-Rouse, 2001), respectively. 4-Fluoro-1,2-dimethoxy-5-(2-propenyl)benzene (RFME) was synthesized following Khrimian et al. (2009). 4-(3,3-Difluoro-2-propenyl)-1,2-dimethoxybenzene (DFME-2) and 4-(3-fluoro-2-propenyl)-1,2-dimethoxybenzene (FME) were prepared following Khrimian et al. (2006).

*1-(3,3-Difluoroprop-2-en-1-yl)-2-fluoro-4,5-dimethoxybenzene (**I**)* To a solution of RFME (3.92 g, 20 mmol) in ether (200 ml) was added a solution of OsO_4 (1.017 g, 4 mmol) in acetone (40 ml). The resulting dark-brown solution was

stirred for 10 min. prior to addition of water (200 ml). Finely powdered $NaIO_4$ (19.2 g, 0.09 mol) was added in five portions over a 2-h period, and the reaction progress was followed by TLC. The mixture was stirred for 1 h, diluted with ether (100 ml), and the layers separated. The organic phase was washed with brine, dried (Na_2SO_4), and purified by flash chromatography on SiO_2 (hexanes/ethyl acetate, 2:1) to yield 2-fluoro-4,5-dimethoxyphenylacetaldehyde (2.12 g, 54%). GC-MS (*m/z*, %): 198 (M^+, 59), 169 (100), 153 (63), 125 (20). A solution of this aldehyde (1.922 g, 9.7 mmol) in THF (10 ml) was added at room temperature to a Wittig reagent, prepared by addition of dibromodifluoromethane (1.78 ml, 19.4 mol) to a solution of hexamethylphosphorus triamide (HMPT, 6.98 ml) in dry THF (40 ml) at 0°C. The resultant mixture was gradually warmed to room temperature, stirred for 1.5 h, then poured into ice-water, and extracted with hexanes/ether (1:1). The organic layer was washed with water, dried, and evaporated. Flash chromatography with hexanes/ethyl acetate (5:1) furnished benzene **I** (1.530 g, 62% from 2-fluoro-4,5-dimethoxyphenylacetaldehyde). GC-MS (*m/z*, %): 232 (M^+, 100), 217 (26), 201 (30), 189 (9), 169 (12), 145 (12), 139 (10), 127 (12), 77 (33). ^{1}H NMR (500 MHz, CD_3CN): 3.25 (br. d, *J*=8.0, CH_2), 3.75 (s, 6 H, OCH_3), 4.50 (dtd, *J*=25.5, 8.0, 2.5, CH=), 6.71 (d, *J*=11.5, H-3), 6.77 (d, *J*=7.4, H-6).

*1-Fluoro-2-(3-fluoroprop-2-en-1-yl)-4,5-dimethoxybenzene (**II**)* A solution of trifluoroanalog **I** (928 mg, 4 mmol) and Red-Al® (3.6 ml of 3.34 M in toluene, 12 mmol) in dry toluene (15 ml) was refluxed, and the reaction progress monitored by GC-MS. After 44 h, the mixture was diluted with ether (50 ml), cooled to −10°C, acidified with 10% HCl to pH 3, and extracted with ether/hexane (1:1). Combined organic extracts were washed with brine, dried, and concentrated. Flash chromatography with hexanes/ethyl acetate (5:1) afforded benzene **II** (632 mg, 74%) in a 89:11 ratio of *E* and Z isomers. GC-MS (*m/z*, %) **II***E*: 214 (M^+, 100%), 199 (30), 183 (37), 151 (12), 139 (14), 137 (15), 127 (23), 109 (12), 59 (23). **II***Z* had an almost identical mass-spectrum. ^{1}H NMR (400 MHz, CD_3CN): 3.18 (br.d, *J*=8.0 Hz, CH_2 **II***E*), 3.37 (br.d, 2 H, *J*=7.2 Hz, CH_2 **II***Z*), 3.75 and 3.76 (both s, CH_3O, **II***E* and **II***Z*), 4.99 (dtd, *J*=42.8, 8.0, 4.8, $CH_2C\underline{H}$=, **II***Z*), 5.58 (ddt, *J*=18.4, 11.0, 8.0, $CH_2C\underline{H}$=, **II***E*), 6.69 (ddt, *J*=85.6, 11.2, 1.2, CH_2CH=$C\underline{H}F$, **II***E*), 6.71 (d, *J*=11.2, H-3, **II***E* and **II***Z*), 6.76 (d, *J*=7.6, H-6, **II***E* and **II***Z*). 6.51 (dt, *J*=4.4, 1.2, 1/2 CH_2CH=$C\underline{H}F$, **II***Z*; the other half of this signal split on F with $^2J_{HF}$~85 Hz was buried under other signals).

*5-Fluoro-4-(3-fluoroprop-2-en-1-yl)-2-methoxyphenol (**III**)* To a solution of **II** (39 mg, 0.18 mmol) in dry DCM (200 μl) was added, under N_2 atmosphere, boron trichloride (0.2 mmol, 200 μl of 1.0 M in DCM) at 0°C. The solution was allowed to warm to 25°C and stirred at that temperature for 4 h. The mixture was quenched with ice water (2.0 ml) and extracted with DCM (total 10 ml). The combined organic extracts were washed with a sodium bicarbonate solution to pH 7, then dried and concentrated. Flash chromatography with hexanes/ethyl acetate (3:1) provided a mixture of phenols (20 mg) that, according to GC-MS, consisted of four peaks in a 2.8:34.3:5.1:55.9 ratio, assigned by GC-MS and NMR as **IV***Z*, **IV***E*, **V***Z*, and **V***E*, respectively. GC-MS (*m/z*, %): **IV***E*: 200 (M^+, 100%), 185 (10), 167 (21), 165 (16), 155 (8), 140 (12), 137 (17), 127 (8), 109 (25). Phenols **IV***Z*, **V***E*, and **V***Z* have very similar mass spectra to **IV***E*. ^{1}H NMR (400 MHz, CD_3CN): 3.12 (br.d, *J*=8.0 Hz, CH_2 **V***E*), 3.16 (br.d, *J*=7.5 Hz, CH_2 **IV***E*), 3.32 (br.d, *J*=7.6 Hz, CH_2 **V***Z*), 3.35 (br. d, *J*=7.6 Hz, CH_2 **IV***Z*), 3.80 (br. s, OCH_3), 4.97 (dtd, *J*=42.8, 7.6, 4.4, $CH_2C\underline{H}$=, **IV***Z*, **V***Z*), 5.50 (m, $CH_2C\underline{H}$=, **IV***E*, **V***E*), 6.32 (br. s, OH), 6.57 (d, *J*=10.8, H-6, **III**), 6.66 (d, *J*=7.6, H-6, **IV**), 6.69 (ddm, *J*=85.6, 10.8, CH_2CH=$C\underline{H}F$, **IV***E*, **V***E*), 6.71 (d, *J*=11.2, H-3, **IV**), 6.76 (d, *J*=7.2, H-3, **III**).

*4-(3,3-Difluoroprop-2-en-1-yl)-5-fluoro-2-methoxyphenol (**V**)* Analogous to the experiment with difluoroanalog **II**, a solution of **I** (40 mg, 0.17 mmol) in dry DCM (200 μl) was treated with boron trichloride (0.2 mmol, 200 μl of 1.0 M in DCM). After the work up described above and flash chromatography with hexanes/ethyl acetate (3:1), we isolated a mixture of phenols **V** and **VI** (24 mg) in a 42:58 ratio, according to GC-MS. GC-MS (*m/z*, %): **V**—218 (M^+, 100%), 203 (16), 187 (12), 186 (10), 183 (17), 167 (11), 158 (10), 155 (32), 127 (35), 77 (10). **VI**—218 (M^+, 100%), 203 (6), 201 (12), 186 (8), 183 (12), 167 (10), 158 (18), 155 (25), 127 (28), 77 (11). ^{1}H NMR (500 MHz, CD_3CN): 3.21 (br.d, *J*=8.0, CH_2, **VI**), 3.24 (br.d, *J*=8.0, CH_2, **V**), 3.80 (br. s, OCH_3), 4.47 (dtd, *J*=25.5, 8.0, 2.0, $CH_2C\underline{H}$=), 6.34 (br. s, OH), 6.59 (d, *J*=10.5, H-6, **V**), 6.68 (d, *J*=7.5, H-6, **VI**), 6.72 (d, *J*=11.2, H-3, **VI**), 6.78 (d, *J*=7.3, H-3, **V**).

Insects Laboratory-reared *B. dorsalis* (strain: "Punador", initiated in 1988–1989 and monitored by mating and survival tests) were obtained from the USDA-ARS-PBARC rearing facility in Honolulu, Hawaii. Pupae were placed in a screened aluminum cage (30×30×30 cm) and allowed to emerge. Flies were given water, sugar, and hydrolyzed yeast protein and held at 25–26°C, 50–70% relative humidity and a 12/12 hL/D cycle before use in laboratory trials. Field bioassays were conducted with sterile laboratory-reared *B. dorsalis*, obtained as pupae, from a laboratory colony at the USDA-ARS-PBARC rearing facility in Honolulu, HI. Emerged adults were kept in an insectary at 24–27°C, 65–70% RH, and a photoperiod of 12:12 h (L:D). Adults were fed water and a diet of three parts sucrose, one part protein yeast hydrolysate (Enzymatic,

United States Biochemical Corporation, Cleveland, OH, USA), and 0.5 part torula yeast (Lake States Division, Rhinelander Paper Co., Rhinelander, WI, USA). Sexually mature, 10- 11-d-old flies were used in the field studies.

Bioassays

Laboratory Feeding and Metabolism A single laboratory experiment was conducted to assess the relative behavioral responses (including attraction and feeding) of male *B. dorsalis* to trifluoroanalog **I**, difluoroanalog **II**, ME, and a water control. Additionally, flies from this experiment were extracted and analyzed to determine phenylpropanoid consumption and metabolism. The lab experiment was conducted using 200 laboratory-reared mature (9-10-d-old) *B. dorsalis* males placed in 20 ~1-l plastic containers (10 flies/container) equipped with water inserts and screened covers. Fifty flies were used for each of the four treatments. Flies were offered 10 μl of ME, trifluoroanalog **I**, or difluoroanalog **II**, on a slip of filter paper (25×5 mm) taped to a blank water vial. A control group of 50 male flies was presented with a filter paper treated with 10 μl water. Responses of flies to the treated or control filter paper were observed for 1 h, with the number of flies present on the filter papers (landings) recorded at 5 min intervals. After the observation period, the filter papers were removed, water containers were introduced, and the flies left overnight with a sugar cube on the cover for consumption. Numbers of dead flies were counted at 18, 24, 36, and 48 h following the observation period. Eighteen hours after feeding, the rectal glands of 10 flies, each fed with ME difluoroanalog **II** and trifluoroanalog **I**, as well as 10 control flies, were dissected and placed in conical plastic vials, each containing 20 μl ethanol. The glands were crushed with a wire rod, sealed with Parafilm, and centrifuged at~4000 rpm for 10 min. The supernatants were diluted with ethanol to 100 μl, and analyzed by GC-MS in splitless mode. External standards of CA, DMP, and phenols **III** and **V**, were used for quantification.

The remainder of the dissected flies were placed individually into 20 μl ethanol, crushed, and then flash-chromatographed on a short SiO_2 column (30×5 mm) with hexanes/ethyl acetate (1:1). The fractions containing unmetabolized ME, and fluorine analogs **I** and **II**, were combined, concentrated to 1 ml, and quantified by GC using external standards.

Field Attractiveness and Release Rate

Dispenser Preparation and Field Trapping A field experiment was conducted to assess the attraction of male *B. dorsalis* to various phenylpropanoids. Lures from this experiment were extracted and analyzed to determine lure longevity. The following compounds were tested in this field trapping experiment: ME, trifluoroanalog **I**, difluoroanalog **II**, RFME, DFME, and FME. Cotton dental wicks (9 mm diam.×14 mm length) were treated with aliquots of a hexane-acetone stock solution (2:1 v/v) containing a test compound. Each wick (total 17 for each treatment) was treated with 250 μl of the stock solution, a volume calculated to deliver ca. 10 mg of a test chemical. After allowing the solvent to evaporate (20–25 min), 3 wicks were kept for day 0 analyses, and the remaining 14 wicks sealed in vials and sent to Hawaii for field testing. Wicks were enclosed by screen material (to prevent feeding) and placed under tarpaulins for trapping and weathering studies. Using metal clips, baskets containing wicks were hung inside Jackson traps. Each Jackson trap had a sticky basal insert and half a Vaportape II pest strip (2.54×10.16-cm strip, weighing 5.9 g, containing 10% 2,2-dichlorovinyl dimethyl phosphate, Hercon Environmental, Emigsville, PA, USA). Field tests were conducted in a macadamia (*Macadamia integrifolia* Maiden and Betche) orchard near Hilo, Hawaii. A natural low-density population of wild Oriental fruit flies, subsisting on wild hosts inside and outside this orchard, was used. The experimental design was a randomized complete block with eight replications (day 1 had 14, d 2 had 11, and d 7 had 8 replicates). For weathering analysis (see below), 3 wicks were retrieved on d 1, d 2, and d 7. Traps were placed 10 m apart in each row, with 20 m between rows. After 24 and 48 h, inserts with trapped adults from each trap were recovered, and new inserts placed in the traps (3 lures were also removed each time, see below). After 7 d, the remaining wicks and inserts were recovered from each trap and brought back to the laboratory. The total numbers of trapped *B. dorsalis* males were determined for each retrieved insert.

Weathering of Wicks and Residue Analyses Concurrent with the attractancy field tests (above), three wicks were retrieved on d 1, 2, and 7. Wicks were placed in glass vials and kept in a freezer. On d 1, replicates 3, 8, and 11 were retrieved for analysis. On d 2, replicates 4, 7, and 12 were retrieved for analysis. On d 7, 3 wicks were randomly selected from the remaining eight replicates left in the field. Upon completion of the experiment, weathered wicks were sent to the ARS lab in Beltsville for analysis. Wicks were extracted in 15 ml of acetone for 12 h and analyzed using appropriate external standards.

Data Analysis All results were analyzed using ANOVA followed by Student Newman–Keuls test (alpha=0.05). Means of fly landing and mortality were compared at each time interval and also combined across an experi-

ment. Lure weathering residual analysis data showed first order exponential decay; lure volatilities are, therefore, reported as half-lives. All statistical analyses were performed using SPSS version 15.0 (SPSS, Inc. Chicago, IL, USA).

Results

The syntheses of trifluorinated and difluorinated methyl eugenol analogs **I** and **II**, as well as phenols **III** and **V**, are shown in Scheme 1a. The oxidative cleavage of the double bond of RFME, following Benbow and Katoch-Rouse (2001), furnished intermediate 2-fluoro-4,5-dimethoxyphenylacetaldehyde, which was difluoroolefinated *in situ* with HPMT/CBr_2F_2 (Hayashi et al., 1979) to give trifluoroanalog **I**. Selective reduction of **I** with Red-Al® gave predominantly **II***E* difluoroanalog (**II***E*/**II***Z*, 87:13) as expected (Hayashi et al., 1979; Khrimian et al., 2006). Cleavage of both difluoroanalog **II** and trifluoroanalog **I** proceeded non-regioselectively, providing mixtures of phenols (Scheme 1a), in which desired phenols **III** and **V** were minor components. Structures of these phenols were assigned without separation from major components by applying nuclear Overhauser effect (NOE), analogously to that previously reported (Khrimian et al., 2009). In the case of the difluoroanalogs mixture, irradiation of CH_3O groups, resonating at about the same frequency in both **III** and **IV**, resulted in a NOEDIF spectrum with enhanced signals at 6.71 (d, J=11.2 Hz) and 6.76 (d, J=7.2 Hz). Based on the literature evidence of a larger proton-fluorine coupling constant in the *ortho* position ($^3J_{HF}$), compared to that in the *meta* position ($^4J_{HF}$) and the actual values of these interactions (Silverstein et al., 1980), we assigned the first doublet (of higher intensity) to the proton on C-3 of phenol **IV**, and the second to the proton on C-3 of phenol **III** (Scheme 1a). In the ^{1}H NMR spectrum of the mixture of **V** and **VI**, NOE effects from CH_3O groups were observed on the signals at 6.72 (d, J=11.2 Hz) and 6.78 (d, J=7.3 Hz). Thus, the bigger doublet at 6.72 ppm with larger coupling constant was assigned to H-3 proton of phenol **VI**, and the smaller doublet at 6.78 to H-3 of phenol **V**. Retention times and mass spectra of synthetic phenols **III** and **V** matched those of compounds isolated from glands of *B. dorsalis* males fed with difluoroanalog **II** and trifluoroanalog **I**, respectively (Figs. 1 and 2).

Feeding and Metabolism Observed responses of male *B. dorsalis* to phenylpropanoid treated filter papers included, flying to and alighting on the paper, arrestment on the paper without feeding, and arrestment with mouthparts engaging the paper (feeding). All behaviors were similar to those previously observed in this bioassay (Khrimian et al., 2009). Comparing male *B. dorsalis* responses to the phenylpropanoids (Fig. 3), the average numbers of flies observed (landing) on trifluoroanalog **I** or difluoroanalog **II** were both higher (F=7.42; P=0.002) than those on ME at 5 min, and difluoroanalog **II** landings and feeding were also higher (F=9.30; P=0.001) than those of ME at 10 min. At 10 min. there was no difference between the responses to fluorine analogs and to ME, but there was at 30 min (F=6.93; P=0.003), when trifluoroanalog **I** no longer appeared to be attractive. When mean landings are combined across the experiment, difluoroanalog **II** landings were higher than those of ME and trifluoroanalog **I**, which were, in turn, higher than the control (F=18.0; P< 0.001). Only one fly landing was observed in the control cages.

Flies fed with trifluoroanalog **I** and difluoroanalog **II** suffered higher levels of mortality than those fed with ME or water (control) (Fig. 4), at each time interval (18 h: F=10.70; P<0.001, 24 h: F=10.95; P=0.001, 36 h: F=8.77; P=0.002, and 48 h: F=9.83; P=0.001). There were no differences in mortality between flies fed on trifluoroanalog **I** or difluoroanalog **II** at any time interval. No fly mortalities were observed in control cages, and only one fly died in ME treatment cages.

Chemical analyses of rectal glands and remaining body parts of 10 flies from the laboratory bioassay showed differences in the total amount of phenylpropanoid intake and metabolites produced by flies (Fig. 5). Representative gas chromatograms of gland extracts of individual *B. dorsalis* males fed trifluoroanalog **I** or difluoroanalog **II** (Figs. 1 and 2) revealed peaks from compounds that matched synthetic phenols **III** and **V** in both retention times and mass spectra. As expected, flies fed with ME produced the well-described metabolites CA and DMP (Fig. 5). Unmetabolized test phenylpropanoids were not found in rectal glands, but were present in extracts of fly remains after dissection. Flies exposed to ME had significantly more unmetabolized phenylpropanoid (ME) than those exposed to trifluoroanalog **I** or difluoroanalog **II** (F=10.93; P<0.001) (Fig. 5). Similarly, a remarkable difference among treatments was observed in the amounts of metabolites relative to amounts of intake, with higher amounts of CA and DMP detected compared to metabolites **III** and **V** (F=10.13; P=0.001). At this feeding level, *B. dorsalis* males were able to metabolize, after 18 h, an average of 13±4 μg/fly of ME (12±3% of ME consumed; CA: 4.46 μg/fly, DMP: 8.10 μg/fly), but only 0.3±0.1 μg/fly of trifluoroanalog **I** (10±5% of consumed), and 0.2±0.1 μg/fly of difluoroanalog **II** (2±2% of consumed). Control flies were not analyzed, as previous work (Khrimian et al., 2006, 2009) had failed to detect phenylpropanoids in the glands or body parts of flies exposed only to water.

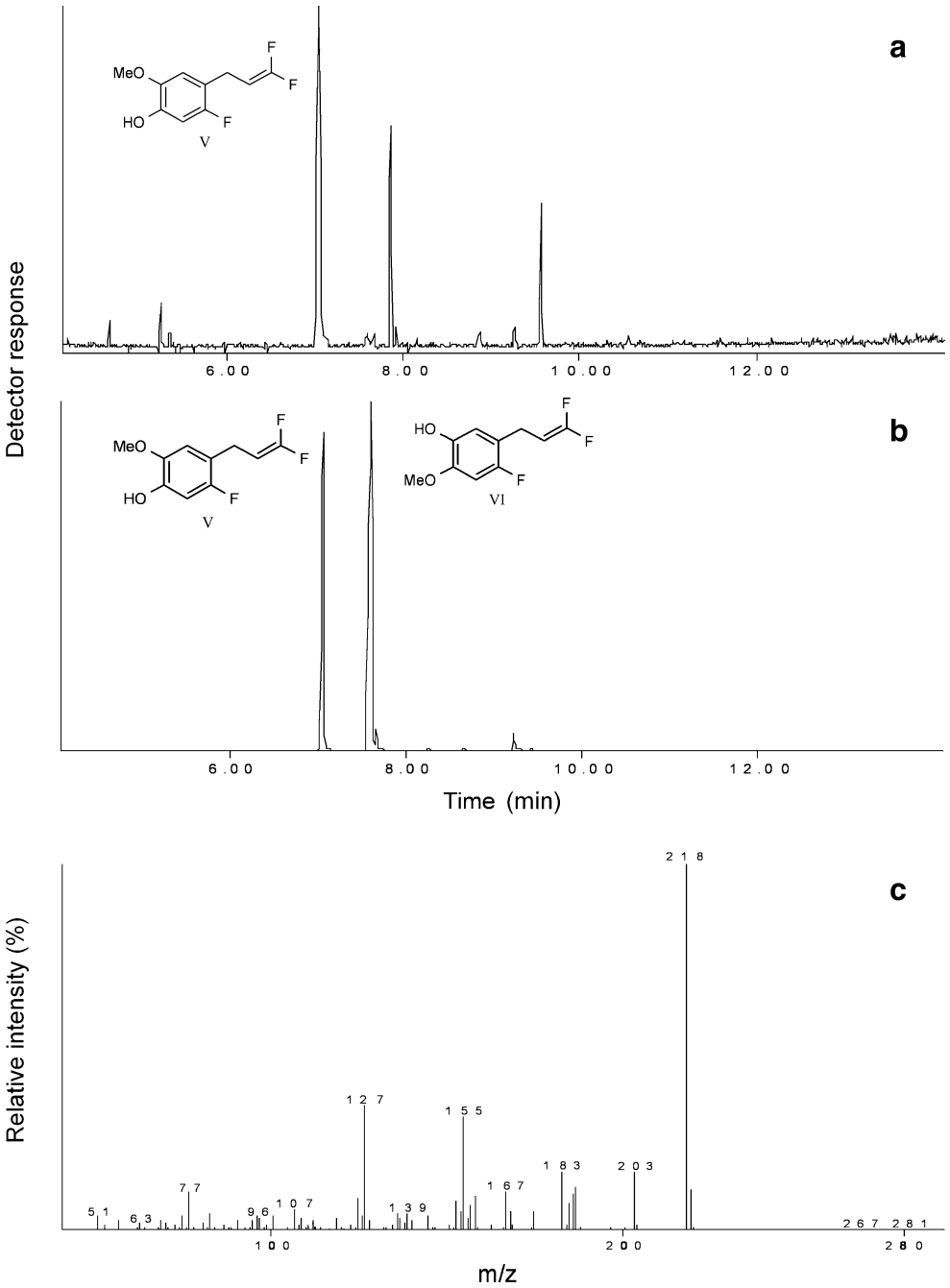


Fig. 1 **a** Representative total ion chromatogram (TIC) of a gland extract of a single *Bactrocera dorsalis* male fed with trifluoroanalog **I**; **b** TIC of a mixture of the synthetic metabolite **V** and isomeric phenol **VI**; **c** Mass-spectrum of the metabolite **V** [4-(3,3-difluoroprop-2-en-1-yl)-5-fluoro-2-methoxyphenol]

Field Bioassay Trap captures of male *B. dorsalis* are presented in Fig. 6. At 24 h (day 1), traps baited with ME and FME captured more flies than traps baited with any other lure, while trifluoroanalog **I**-baited traps caught fewer flies than any of the traps baited with other lures ($P<0.001$, $F=10.627$). At 48 h (day 2), the differences among lures of numbers of flies caught in traps was less pronounced ($P<0.001$, $F=7.10$). At day 7, FME-baited traps caught more flies than all other traps, while RFME-, DFME- or trifluoroanalog **I**-baited traps caught fewer flies than traps baited with other lures ($P<0.001$, $F=26.75$). Overall, traps baited with trifluoroanalog **I** were outperformed by traps baited with any of the other lures, while traps baited with difluoroanalog **II** caught numbers of flies that were always intermediate (Fig. 6).

Analysis of weathered lures showed marked differences in amounts of phenylpropanoid residues (Table 1). RFME had the shortest half-life ($P<0.001$, $F=2.82$; and, therefore, was the most volatile), although it was not different from that of ME. The half-life of FME was longer (and, therefore, the

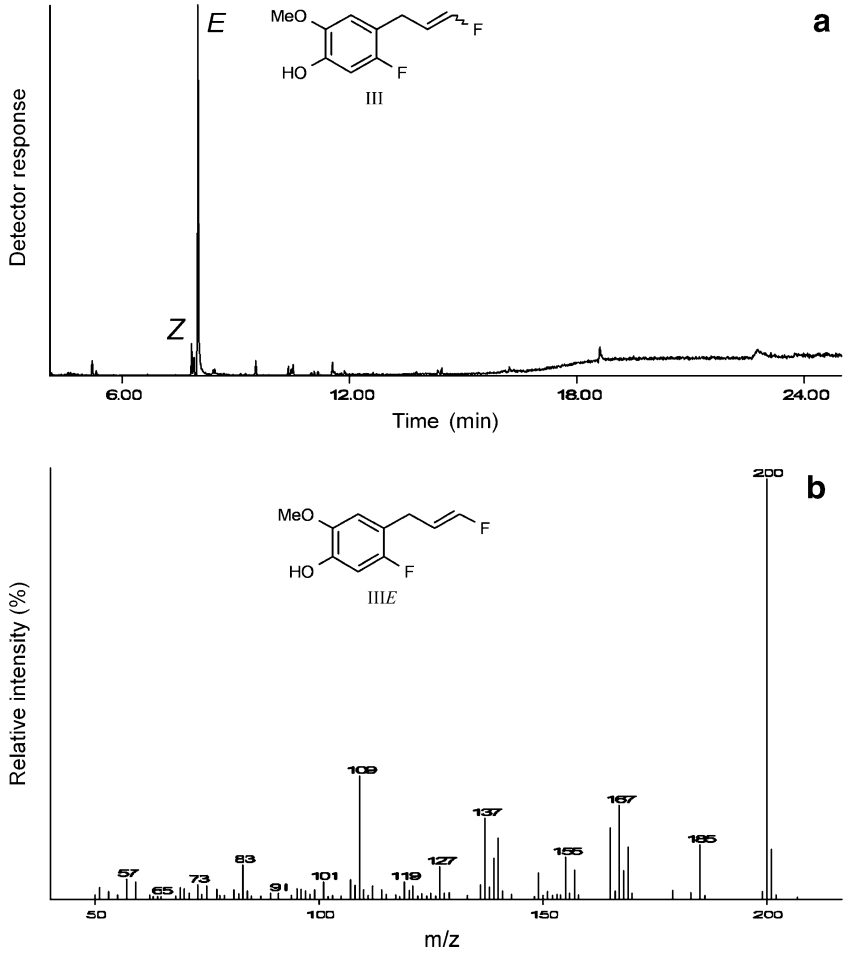


Fig. 2 **a** Representative total ion chromatogram (TIC) of a gland extract of a single *Bactrocera dorsalis* male fed with difluoroanalog **II,** and (**b**) the mass spectrum of the main metabolite **III***E*

least volatile) than for all other phenylpropanoid lures. As a general observation, ring-fluorination decreased half-life time (i.e., increased volatility), while side-chain fluorination increased half-life (i.e., decreased volatility).

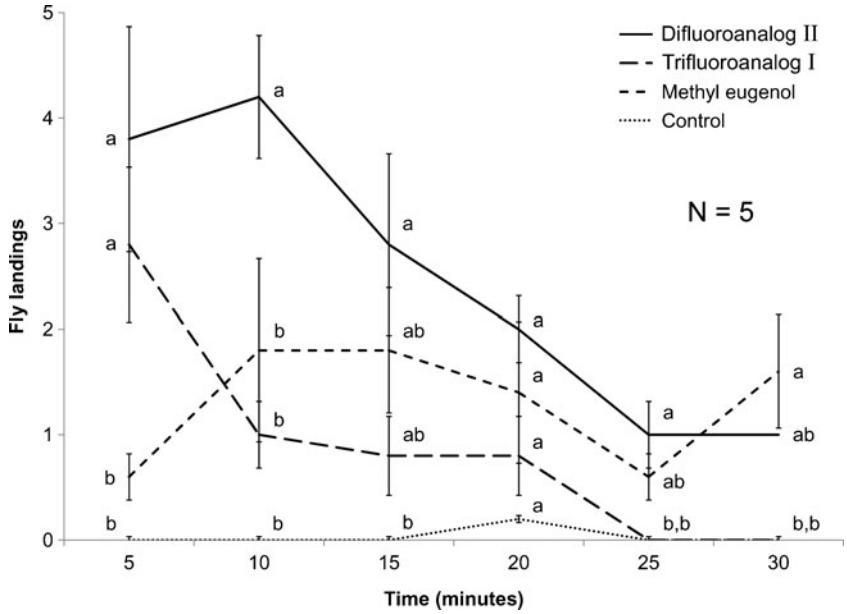


Fig. 3 Mean numbers (± SE) of male *Bactrocera dorsalis* landings every 5 min on papers treated with different chemicals. Different letters represent differences ($P<0.05$) among the mean numbers of fly landings at a given time interval (ANOVA, followed by SNK test)

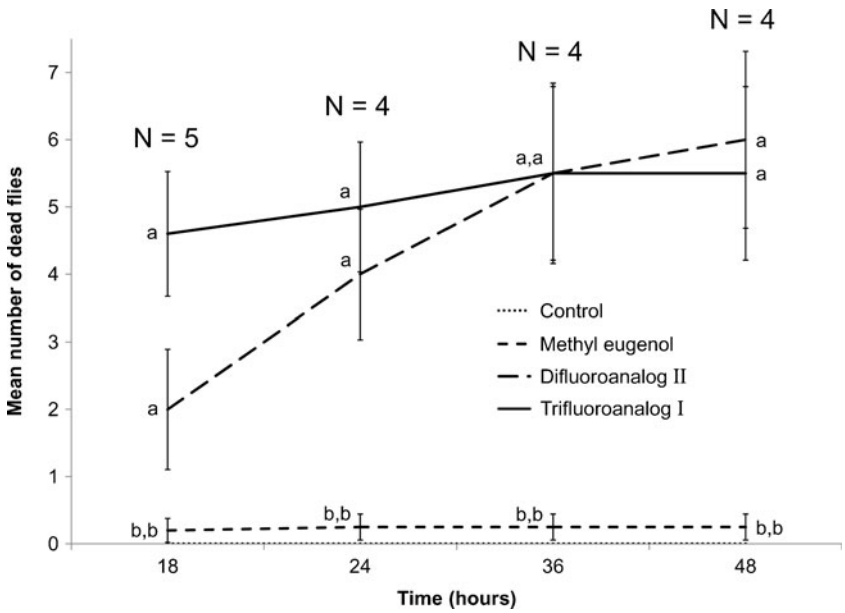


Fig. 4 Mean numbers (± SE) of dead male *Bactrocera dorsalis* at various times after exposure to different chemicals. Different letters represent differences ($P<0.05$) among mean numbers of dead flies at a given time interval (ANOVA, followed by SNK test)

Discussion

Our laboratory studies demonstrated that the di- and trifluorinated analogs **I** and **II** are perceived by *B. dorsalis* males and evoke responses similar to that of the natural phenylpropanoid methyl eugenol. In close-range experiments, flies were attracted to trifluoroanalog **I**, difluoroanalog **II**, and ME in similar numbers, but flies absorbed more ME. Flies did not need to contact the filter papers directly to absorb appreciable amounts of vaporized trifluoroanalog **I**, difluoroanalog **II**, or ME. This is shown by the high concentration of ME found in flies, despite only a moderate number of landings. This is consistent with previous observations of phenylpropanoid intake in *B. dorsalis* (Khrimian et al., 2006, 2009).

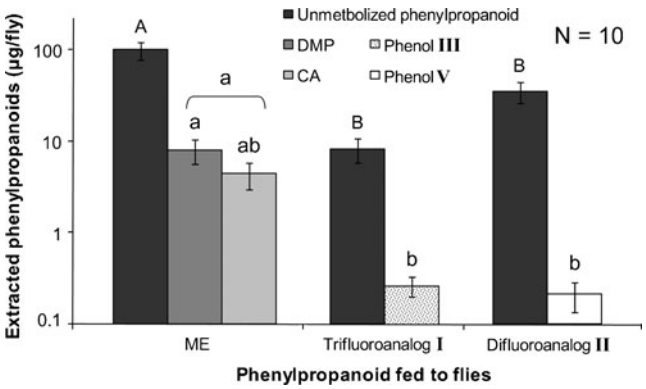


Fig. 5 Mean amounts (± SE) of unmetabolized phenylpropanoids and metabolites in *Bactrocera dorsalis*, 18 h after exposure to various phenylpropanoids. Metabolites **III** and **V** were in rectal glands, while unmetabolized methyl eugenol (ME), difluoroanalog **II** and trifluoroanalog **I** were from other body parts. Different capital letters represent differences ($P<0.05$) among amounts of unmetabolized phenylpropanoids, while different lowercase letters represent differences among amounts of phenylpropanoid metabolites (ANOVA, followed by SNK test). ME metabolites, (*E*)-coniferyl alcohol (CA) and 2-(2-propenyl)-4,5-dimethoxyphenol (DMP), were grouped to allow comparison of total metabolites produced from each phenylpropanoid (shown above bracket)

Introduction of fluorine atoms on both the ring and side-chain of ME blocks both positions that undergo enzymatic hydroxylation, all but completely inhibiting these phenylpropanoid biotransformation pathways. While this was not particularly surprising for trifluoroanalog **I**, it is interesting that difluoroanalog **II** did not undergo hydroxylation on the side-chain as does FME (Khrimian et al., 2006), which shares a mono-fluorinated side-chain moiety with **II**. The terminal double bond of **I** appears to be more resistant to enzymatic hydroxylation than either **I** or FME, but there is little structural difference between these two ME analogs. It seems likely that ring fluorination, via inductive and/or resonance effects, completely inhibits side-chain hydroxylation in **II**. Although enzymatic hydroxylation was blocked in **I** and **II**, two new metabolites were produced through demethylation of the methoxy group positioned para to the side-chain. This *O*-demethylation is part of the metabolic pathway leading to the formation of coniferyl alcohol from ME or FME (Khrimian et al., 2006). However, in the formation of phenols **III** and **V**, this *O*-demethylation appears to operate independently of oxidative biotransformations. Additionally, it is interesting to note that almost equal amounts of trifluoroanalog **I** and difluoroanalog **II** were metabolized (Fig. 5), in spite of differences in intake, suggesting that there is a finite limit to the amount of di-/trifluorinated phenylpropanoid that can be metabolized. The amounts of trifluoroanalog **I** and difluoroanalog **II** metab-

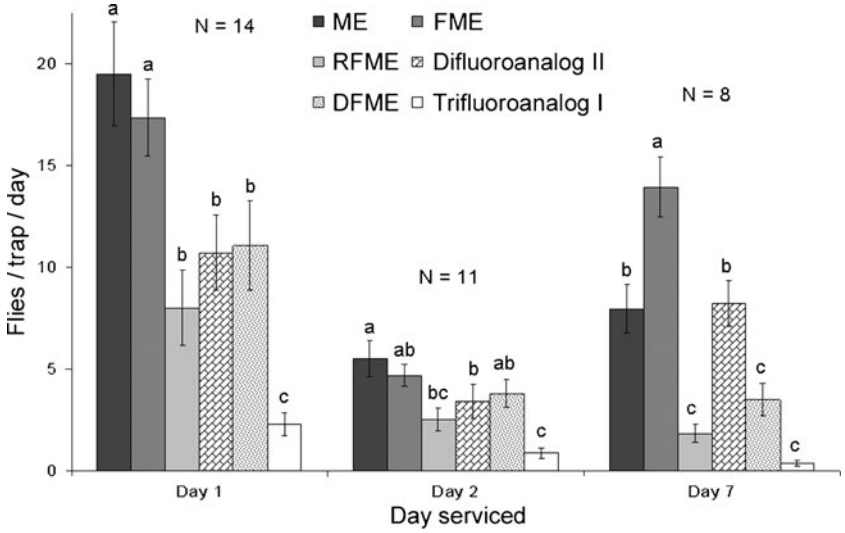


Fig. 6 Mean numbers (± SE) of male *Bactrocera dorsalis* caught per trap in a macadamia orchard near Hilo, Hawaii. Different letters represent significant differences ($P<0.05$) among treatments at a given time interval (ANOVA, followed by SNK test). Differences in the replicate number reflect the removal of treated lure wicks for analysis. ME=methyl eugenol; RFME=1-fluoro-4,5-dimethoxy-2-(prop-2-en-1-yl) benzene; DFME=4-(3,3-difluoroprop-2-en-1-yl)-1,2-dimethoxybenzene; FME=4-[(2*E*)-3-fluoroprop-2-en-1-yl]-1,2-dimethoxybenzene

olized were less than those of FME and RFME (Khrimian et al., 2006, 2009). However, the amount of fluorinated ME consumed by *B. dorsalis* males varied for each analog, with **I** and **II** being the least taken in.

Intake (consumption or vapor absorption) of both trifluoroanalog **I** and difluoroanalog **II** resulted in increased mortality in *B. dorsalis* males compared to flies fed with ME or water (control). ME has been reported to cause death in the Oriental fruit fly when ingested in large amounts (Steiner, 1952). Thus, the fitness of *B. dorsalis* males is evidently linked (among other factors) to their ability to metabolize phenylpropanoids efficiently, as consumption appears to be compulsive. The significant inhibition of fluorinated phenylpropanoid metabolites in *B. dorsalis* appears to be caused by complete blocking of the key metabolic hydroxylation sites in trifluoroanalog **I** and difluoroanalog **II**. Hydroxylation often is the first step (Phase I) in the detoxification pathways used by insects (Dauterman and Hodgson, 1978). Given the reported toxicity of ME to *B. dorsalis*, when consumed in large quantities, (Steiner, 1952), it is possible that trifluoroanalog **I** and difluoroanalog **II** interrupt 'normal' phenylpropanoid metabolism and thereby cause increased mortality. However, blocking metabolism, with a resultant buildup of unmetabolized phenylpropanoid, does not in itself explain the higher mortality from **I** and **II** compared to ME. ME-exposed flies had much higher overall levels of phenylpropanoids in their bodies when analyzed, suggesting that **I** and **II** may also be more inherently toxic to *B. dorsalis*.

Attract-and-kill techniques have been used to control a number of pest insects, including fruit flies (El-Sayed et al., 2009). In this technique, pests are attracted to a point source by a semiochemical lure and die after being exposed to a killing agent. In most cases, the semiochemical lure and killing agent are different compounds. The toxicity of trifluoroanalog **I** and difluoroanalog **II** suggests an alternative, in which a single compound may function as both attractant and killing agent. Many specialist insect herbivores use plant secondary compounds as attractants and feeding stimulants (Metcalf and Metcalf, 1992; Harborne, 1993). These compounds often are toxic to generalist insect herbivores, while the specialists have circumvented toxic effects through either behavioral or biochemical mechanisms. In the case of ME, *B. dorsalis* appears to be physiologically tolerant of this phenylpropanoid, with mortality occurring only after large amounts have been ingested (Steiner, 1952). The molecular modifications of trifluoroanalog **I** and difluoroanalog **II** cause increased mortality of *B. dorsalis*, while retaining some attractant and phagostimulatory activity toward *B. dorsalis*. However, the moderate level of *B. dorsalis* mortality caused by trifluoroanalog **I** and difluoroanalog **II** in this study is unlikely to be useful for control (especially compared to the current methods of inclusion of a toxicant with ME). This concept, of modifying attractive/phagostimulatory semiochemicals to be toxic to insects based on specific knowledge of attractant/consumption metabolism, may, however, be applicable to other pests.

Although *B. dorsalis* males were attracted strongly to, and fed on, trifluoroanalog **I** and difluoroanalog **II** in a field cage experiment, the captures of male oriental fruit flies to traps baited with either of these compounds was markedly

lower than to traps baited with ME. Traps baited with trifluoroanalog **I** captured roughly 10% of the number of flies caught in traps baited with ME at similar release rates. Traps baited with difluoroanalog **II** captured roughly half the number of flies caught in traps baited with ME on day 1, but by day 7 catches were roughly equal (overall **II** captured two thirds that of ME captures). The release rate of difluoroanalog **II** is significantly less than that of ME, suggesting at similar release rates that their catches could be similar. We did not determine the release rate of DFME, but traps baited with this compound captured roughly half the number of flies caught in traps baited with ME.

Metcalf et al. (1975; 1981) studied the molecular parameters of attractants and olfaction in *B. dorsalis* that involved a number of substituted orthodimethoxybenzenes. Two chemical structure postulates were derived from those studies: (1) unshared electron pairs of the orthodimethoxy groups were essential for receptor depolarization, yet the methoxy group in the para position to the allyl group of ME is of primary importance; (2) electron-donating side-chain groups produced stronger attractants than those withdrawing electrons. We did not find in the literature any activity correlations with the substitution pattern of trifluoroanalog **I** and difluoroanalog **II**, but because the fluorine atom is isosteric with hydrogen (and being such has been widely pursued for altering the properties of biologically active substances), it seems that it is the electronic factor of fluorine substitution that is responsible for the reduced attractiveness of trifluoroanalog **I** and difluoroanalog **II** compared to ME. A strong electronegative effect of the fluorine atom should decrease electron density about the CH_3O groups in **I** and **II** but would primarily affect the methoxy group in the para-position to F (meta to allyl group), thus diminishing, but not abolishing, the attraction to *B. dorsalis*. In any case, a moderate (**II**) or poor (**I**) ability to attract flies in the field, and likely higher costs of syntheses, make trifluoroanalog **I** and difluoroanalog **II** unlikely replacements for ME as attractants for the Oriental fruit fly. Our field tests, however, showed good catches in traps baited with FME, confirming our earlier findings (Khrimian et al., 1994, 2006, 2009; Liquido et al., 1998) that this compound is equally attractive to *B. dorsalis* and also a longer-lived lure.

In summary, we developed tri- and difluorinated analogs of methyl eugenol that were attractive to *B. dorsalis* males in a laboratory cage experiment, but which were only moderately (**II**) or poorly (**I**) attractive in field trials. These new fluorine analogs are metabolized more slowly than ME after being consumed by *B. dorsalis* males. As a result of blocking the key enzymatic hydroxylation sites, both compounds undergo enzymatic demethylation of the methoxy group positioned para to the side-chain.

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Attraction and Oviposition of *Tuta absoluta* Females in Response to Tomato Leaf Volatiles

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Abstract The tomato leafminer *Tuta absoluta* (Lepidoptera: Gelechiidae) is a devastating pest of cultivated tomato *Solanum lycopersicum* throughout South and Central America and Europe. We aimed to characterize the behavioral mechanisms and the chemical cues involved in host selection of *T. absoluta* females by chemical analysis of tomato leaf volatiles, wind tunnel attraction assays, and oviposition bioassays. Tomato leaf odor elicited in mated females upwind orientation flight followed by landing as well as egg-laying, demonstrating the essential role of plant volatiles in *T. absoluta* host-finding behavior. In wind tunnel and oviposition choice experiments, *T. absoluta* females significantly preferred tomato *S. lycopersicum* over wild tomato *Solanum habrochaites*, which is resistant to larval feeding. This indicates that leaf volatiles provide information on the suitability of plants as larval hosts. Mated females also discriminated three cultivars of *S. lycopersicum* according to their volatile profiles. Headspace collections from leaves of these three cultivars contained large amounts of β-phellandrene, followed by limonene, 2-carene, and (*E*)-β-caryophyllene, which together accounted for more than 70% of tomato foliage headspace. Most leaf volatiles were released by all three cultivars, but they showed significant differences with respect to the presence of a few minor compounds and blend proportion. This is an initial study of the volatile signatures that mediate attraction and oviposition of tomato leafminer *T. absoluta* in response to its main host, tomato.

Key Words *Tuta absoluta* · Host plant volatiles · Kairomone · Headspace collection · Chemical analysis · *Solanum lycopersicum* · Lepidoptera · Gelechiidae

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Introduction

Plants release a variety of volatile organic compounds that play multiple roles in interactions with other plants and animals (Dudareva *et al.*, 2004; Knudsen *et al.*, 2006; Pichersky *et al.*, 2006). Insect herbivores exploit these volatiles to locate their host plants at a distance, for feeding, mating, and egg-laying (Linn *et al.*, 2003; Bengtsson *et al.*, 2006; Tasin *et al.*, 2006; Pinero and Dorn, 2009; Cha *et al.*, 2008; Schmidt-Busser *et al.*, 2009; Sole *et al.*, 2010). In addition to olfaction, vision and contact chemoreception (taste) could play a role at a short distance (Renwick and Chew, 1994; Calatayud *et al.*, 2008; Pontes *et al.*, 2010). Knowledge of the chemicals and mechanisms that mediate host plant location by insect herbivores is essential for our understanding of plant—insect relationships, and also will lead to the development of novel tools for insect management (Szendrei and Rodriguez-Saona, 2010; Witzgall *et al.*, 2010).

Plant volatiles are products of diverse metabolic pathways, but many are derived from the isoprenoid or terpenoid pathways (Sacchettini and Poulter, 1997; Degenhardt *et al.*,

2009). Solanaceous plants, like tomato, produce a suite of terpenes that likely serve as defense agents against herbivores (Kennedy, 2003; Bleeker *et al*., 2009; Kang *et al*., 2010). The constitutive headspace of undamaged plants varies with genotype, phenological stage, and environmental conditions. It is conceivable that insects use the volatile signals that correlate with this variation to distinguish the most suitable hosts (Bengtsson *et al*., 2001; Vallat and Dorn, 2005; Karlsson *et al*., 2009).

The tomato leafminer *Tuta absoluta* (Meyrick) (Lepidoptera, Gelechiidae) feeds on several solanaceous species, and preferentially on tomato *Solanum lycopersicum* (Siqueira *et al*., 2000). It is a serious threat to commercial tomato production in South and Central America. Since its introduction, it has become an economically important insect in Europe (Torres *et al*., 2001; Desneux *et al*., 2010). It is anticipated that *T. absoluta* will even spread from Central America into Mexico and the USA. Control of *T. absoluta* relies on multiple insecticide sprays, but resistance to these chemicals is growing (Siqueira *et al*. 2000; Lietti *et al*., 2005). Synthetic sex pheromones are used to monitor population levels, but also for control by mass trapping of males in greenhouses (Salas, 2004; Witzgall *et al*., 2010).

Tomato plants are infested at any developmental stage, with females ovipositing preferentially on leaves. After hatching, larvae feed on leaf parenchyma tissue, on tender portions of the stems (especially axillary buds), and in developing and mature fruit, causing bud drop, fruit malformation, fruit rot, and a drastic reduction in leaf area (Torres *et al*., 2001). No tomato cultivars are entirely resistant to *T. absoluta*, but not all cultivars are equally susceptible (Bogorni *et al*., 2003; Oliveira *et al*., 2009). In addition, the wild tomato species *S. habrochaites* (syn. *Lycopersicon hirsutum*) is more resistant to *T. absoluta* and several other tomato herbivores than *S. lycopersicum* (Leite *et al*., 2001; Kennedy, 2003).

The aim of this study was to determine the role of tomato volatiles in host finding and oviposition behavior of tomato leafminer *T. absoluta*. We identified the volatiles released from leaves of three different cultivars of tomato *S. lycopersicum* and investigated the question whether tomato leafminer uses these volatiles for the discrimination of suitable larval hosts.

Methods and Materials

Insect Rearing A laboratory colony of tomato leafminer *T. absoluta* originated from pupae and larvae collected in a commercial tomato plantation located in Viçosa, Minas Gerais (Brazil). The colony was maintained at 24±1°C, 70±10% r.h., and a 12:12 hL:D photoperiod. Adults were fed with sugar water and provided with tomato (cv. Aromata) leaves for egg-laying. Leaf stems were cut and placed through a hole in the transparent plastic lid of a 500-ml recipient containing water and a nutrient solution. Larvae were provided with fresh leaves every 2nd d, and rolls of corrugated paper were added for pupation. Pupae were sexed and placed in plastic cages for eclosion (100×40×40 cm).

Plant Material For behavioral experiments and volatile analysis, three tomato *Solanum lycopersicum* Mill. (Solanaceae) cultivars were used: cv. Santa Clara and cv. Carmen, which are known to be highly susceptible, and cv. Aromata, which is less susceptible (Svalöf-Weibull Trädgård AB, Hammenhög, Sweden) (Bogorni *et al*., 2003; Oliveira *et al*., 2009; Lima *et al*. unpublished data). In addition, the wild tomato species *S. habrochaites*, which is resistant to *T. absoluta*, was used for wind tunnel and oviposition bioassays. Tomato seeds were pre-cultivated in rock wool cubes in the greenhouse for 4 week (r.h.: 75%, light intensity: 200 $\mu molm^{-2}$ $s\text{-}^{1}$). Plants were transplanted to a hydroponic system and fertilized daily with a nutrient solution. Standard values (in mM) for the composition of the nutrient solution were: 6.0 $Ca(NO_3)_2$, 8.0 KNO_3, 5.0 $MgSO_4$, 1.0 NH_4NO_3, 2.3 KH_2PO_4 and for micronutrients: 0.4 Fe–EDTA, 0.01 $MnSO_4$, 0.025 H_3BO_3, 0.005 $ZnSO_4$, 0.00075 CuC_{l2},2 H_2O, 0.0005 Na_2MoO_4,and pH 5.8±0.1.

Flight Behavior Bioassay Female attraction to plants was tested in a wind tunnel (flight section 63×63×200 cm; Tasin *et al*., 2006). Filtered air (24 cylinders with active charcoal, 14.5×32.5 cm, Camfil, Trosa, Sweden) was blown into the tunnel by a centrifugal fan (Fischbach GmbH, Neunkirchen, Germany) at 25 cm/s. Outcoming air was aspirated by another fan and cleaned by two additional sets of charcoal filters. The flight section was lit diffusely from the side at 10 lux, and the room was kept at 23±2°C and 40–60% r. h. Cut tomato plants (ca. 35 cm high) were placed into vials with water in the center of the upwind end of the flight tunnel. Two h before the end of the photophase, females were placed individually into cylindrical plastic containers. They were kept until testing in the wind tunnel room. Moth behavior was scored for upwind oriented flight in the center of the wind tunnel (over at least 60 cm from the downwind end) and for approaching tomato plants. By using this method, we tested (1) the attraction of 2-d-old mated (N=190) and virgin (N=150) females to *S. lycopersicum* cv. Aromata, and (2) the attraction of mated female (N=150) to *S. lycopersicum* cv. Santa Clara and *S. habrochaites*.

Oviposition Bioassays Experiments were done with 2-d-old mated females during 24 hr. Females were individually kept in a glass tube (15.0×3.0 cm) covered on both sides with a

plastic film. To keep the females in one side of the tube, it was divided in half with a nylon grid. The female and the tomato leaf were put on the same (N=48) or different sides of the tube (N=48). This experiment was done with the three tomato *S. lycopersicum* cultivars. After 24 h, the eggs laid on the glass wall and the nylon grid were counted.

In choice tests, mated females were kept in a plastic container (12×10 cm) either with one leaf of cv. Aromata, cv. Carmen and cv. Santa Clara (N=81 females), or with one leaf of Santa Clara and wild tomato *S. habrochaites* (N=37 females), and the leaves were scored for eggs after 24 h.

Plant Volatile Analysis Freshly cut plants of the three cultivars of *S. lycopersicum* were confined separately in a 2-L glass jar that was closed with a ground-glass fitting. The cut end of the branch was held in a 10-ml vial of water. A charcoal-filtered airstream was pulled over the plant material from the bottom to the top of the jar, and over a 35-mg Super Q trap (80/100 mesh; Alltech, Deerfield, IL, USA) which was held between plugs of glass-wool in a 4×40 mm glass tube. Before use, traps were rinsed sequentially with 3 ml methanol, ether, and redistilled hexane, after 15 min treatments in ultrasonic baths in ether and hexane, respectively. The air flow was 150 ml/min, exchanging the headspace in the jar 4.5 times/h. Collections were done for 24 h, at 20–22°C and 10–30 lux. The charcoal filter for incoming air and the Super Q trap for outcoming air were connected with glass fittings to the jar. All glassware was heated to 350°C for 10 h before use. After volatile collections, traps were extracted immediately with 0.5 ml hexane (redistilled; LabScan, Malmö, Sweden). Sample volumes were reduced to 50–60 µl, at ambient temperature in Francke-vials with an elongated tip (5 cm× 2 mm i.d.). Samples were stored in sealed glass capillary tubes at −18°C (Bengtsson *et al*., 2001).

Odor samples were analyzed on a gas-chromatograph coupled with a mass-spectrometer (GC-MS). Volatile compounds in trap eluents were injected in aliquots of 2 µl into a Hewlett- Packard 5970 B MS instrument, with electron impact ionization at 70 eV, and interfaced to an HP 5890 GC (Hewlett- Packard, Palo Alto, CA, USA). The GC was equipped with a fused silica capillary column (30 m×0.25 mm) coated with DB-wax (df=0.25 µm; J&W Scientific, Folsom, CA, USA) using helium as carrier gas (35 cm/s). The temperature program was from 35°C (hold 5 min) at 8°C/min to 230°C (hold 5 min). The Kovats retention index (KI) of each compound was calculated. Tentative identification of compounds was based on comparison of matching of mass spectra with the NIST 98 MS and Wiley databases and a reference library built by measuring commercially available standards, and comparison of KIs on similar columns. Identification of some components was confirmed by comparison with mass spectra and GC retention data with those of standards.

Statistical Analysis All statistical analysis was done using R (version 2.8.0; R Development Core Team, www.R-project.org) following the method of Crawley (2005).

The effect of either mating status or test plant (*S. lycopersicum* and *S. habrochaites)*, on the proportion of females, grouped per day, that were attracted to and that landed on the plant was tested with a Generalized Linear Modelling (GLM) under quasibinomial distribution.

Similarly, the effect of contact with the host plant and the effect of the tomato cultivar (Carmen, Santa Clara, Aromata) on the total number of eggs laid in the non-choice bioassays were tested using a GLM under quasipoisson distribution. In the choice experiments, the effect of *S. lycopersicum* cultivars or tomato species on the proportion of eggs laid on leaves was tested using a GLM with quasibinomial distribution. In all analyses, a full model was fitted, from which terms were deleted in a stepwise fashion to obtain the minimal adequate model (MAM). Significance (P<0.05) was assessed by testing the change in deviance after the removal of a term from the model. Differences among treatment levels were examined by contrast analysis. Candidate similarity levels were amalgamated to compose a new model, which was then compared with the previous un-amalgamated model. The new, simplified model was accepted if it did not differ from the previous, more complex model. Amalgamation proceeded until all treatment levels were checked. Models were checked by residual analyses, and eventually corrected for overdispersion as well as for the correctness of the assumed distribution (Crawley, 2005).

Headspace composition was compared among samples by multivariate analyses using R with Vegan and labdsv packages (Oksanen *et al*., 2008; Roberts, 2010). Non-metric multidi-

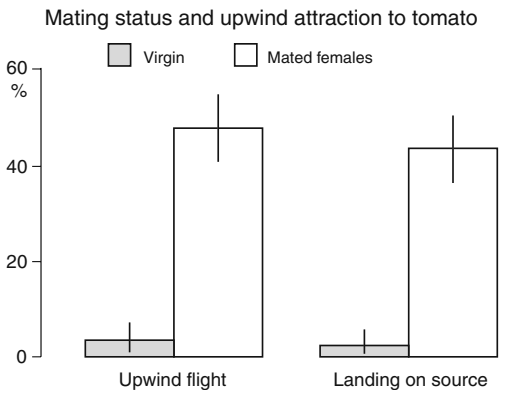


Fig. 1 Proportion of mated and virgin females of tomato leafminer *Tuta absoluta* flying upwind and landing on tomato leaves (*Solanum lycopersicum* cv. Aromata) in the wind tunnel (mean±SE/day). For both behavioral responses, differences between mated (N=190) and virgin females (N=150) are significant (N=17 days, GLM, P<0.05)

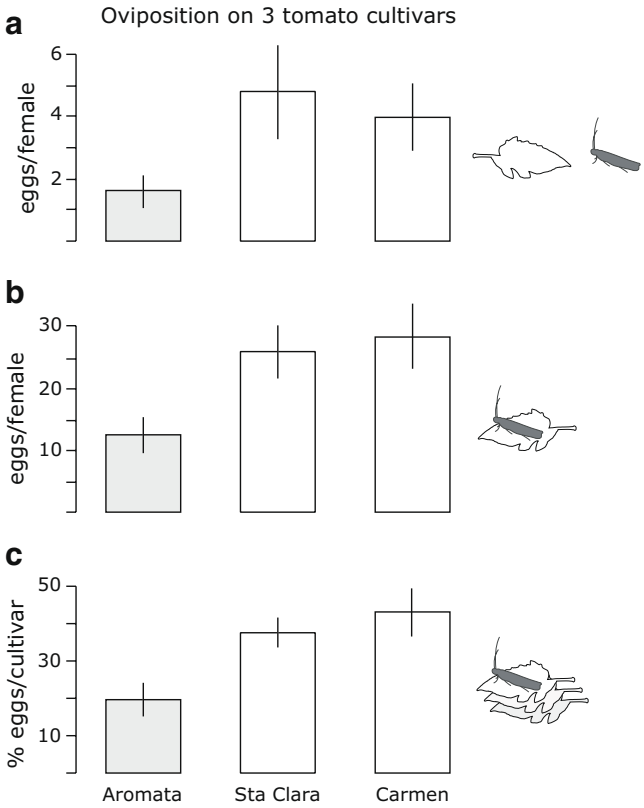


Fig. 2 Oviposition rate (mean±SE) of mated tomato leafminer *Tuta absoluta* females on three tomato cultivars Aromata, Santa Clara, and Carmen, in (**a**) a non-choice experiment without leaf contact (N=48), (**b**) a non-choice experiment with leaf contact (N=48), and (**c**) a choice experiment with leaf contact (N=81). For each experiment, treatments represented by filled and empty bars are significantly different (GLM with contrast, P<0.05)

mensional scaling (NMDS) was used to detect similarities among samples (using amounts relative to total peak area). Data were square-root transformed and standardized before calculating Bray–Curtis similarities (Bray and Curtis, 1957). In oviposition bioassays, *T. absoluta* prefers cv. Santa Clara and cv. Carmen over cv. Aromata with no difference between Santa Clara and Carmen. Thus, based on *T. absoluta* oviposition preference, variation in odor composition among tomato cultivars and between cv. Aromata *vs.* cvs. Santa Clara and Carmen (pooled), was tested for significance using permutational multivariate analysis of variance (permanova) with the Bray–Curtis dissimilarity measurement and 10000 permutations (McArdle and Anderson, 2001).

Differences in the occurrence and abundance of compounds in the headspace of the cv. Aromata *vs.* the cvs. Santa Clara/Carmen were determined according to the method of Dufrêne and Legendre (1997) by calculating 'indicator compound' (*e.g.*, Proffit *et al.*, 2009). For each compound, an observed indicator value (*IV*) was calculated. The deviation of the observed *IV* from a random distribution of *IVs* was tested using a Monte Carlo test with 10000 randomizations. Indicator compounds have an observed *IV* significantly different from the random *IV*, and designate the most characteristic compounds in headspace samples of a group, either because of their occurrence in different samples and/or because of their relative abundance.

Results

Wind Tunnel Experiments Mated tomato leafminer *T. absoluta* females flew upwind over 180 cm towards tomato plants (45% of the females tested) and landed (42%) on these plants. In contrast, unmated females showed no attraction response to tomato (N=17; for flight, $F_{1,15}$=192.63, P<0.001; for landing, $F_{1,15}$=122.09, P<0.001; Fig. 1).

Oviposition Experiments with Tomato Cultivars In non-choice tests, the cultivar had a significant effect on oviposition (df=2, χ^2=132.71, P<0.001; Fig. 2a and b). Tomato leafminer females laid more eggs in response to cvs. Santa Clara and Carmen as compared cv. Aromata (df=1, χ^2=110.39, P<0.001 and df=1, χ^2=95.75, P<0.001), both with and without leaf contact: the interaction between leaf contact and cultivar was not significant (df=2, χ^2=3.38, P=0.82).

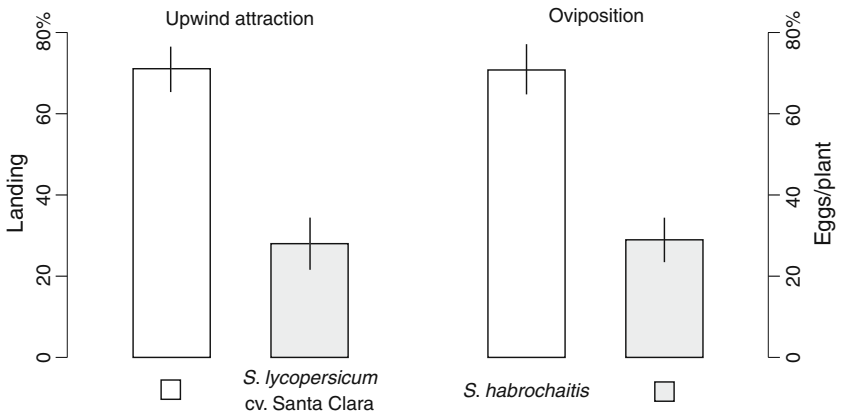


Fig. 3 Upwind attraction (N=22 days, mean±SE/day) and oviposition choice tests (N=37, mean±SE) of mated tomato leafminer *Tuta absoluta* with cultivated *Solanum lycopersicum* cv. Santa Clara and wild tomato *Solanum habrochaites.* The effect of the tomato species was significant in both experiments (GLM, P<0.05)

Table 1 Headspace of tomato foliage of three cultivars Aromata, Santa Clara, and Carmen (*Solanum lycopersicum*). Abundance of volatile compounds (mean±SD), relative to the total amount or released volatiles

Compounds	KI[c]	Aromata *N*=16				Santa Clara *N*=2				Carmen *N*=4				Indicator compounds[e]	
		n[d]	%			n	%			n	%			*IV*	*P*
Green leaf alcohol															
(*Z*)-3-hexen-1-ol[a]	1383	8	0.09	±	0.14	2	0.09	±	0.004						
Aliphatic ketone															
unknown 1	2118	12	0.06	±	0.07					4	0.07	±	0.02		
Monoterpenes															
Hydrocarbons															
β-pinene[a]	1082	13	0.05	±	0.03	2	0.12	±	0.02						
trans-isolimonene	1094	11	0.03	±	0.02	2	0.05	±	0.02						
sabinene[b]	1106	13	0.06	±	0.04	2	0.16	±	0.002						
unknown 2	1116	**16**	**0.35**	**±**	**0.23**	2	0.51	±	0.03	4	0.04	±	0.02	0.67	0.01
2-carene[a]	1123	**16**	**14.94**	**±**	**4.48**	2	16.79	±	0.15	4	3.52	±	0.39	0.67	0.01
3-carene[a]	1138	13	0.04	±	0.03	2	0.07	±	0.00						
unknown 3	1147	7	0.02	±	0.02										
β-myrcene	1154	4	0.76	±	1.28										
α-phellandrene[b]	1157	16	3.66	±	1.00	2	5.25	±	0.23	4	4.24	±	0.28		
α-terpinene[a]	1173	**16**	**2.02**	**±**	**0.49**	2	1.90	±	0.06	4	2.30	±	0.15	0.57	0.01
limonene[a]	1197	16	9.44	±	2.04	2	13.18	±	5.71	4	10.09	±	2.19		
β-phellandrene [b]	1211	**16**	**51.87**	**±**	**6.28**	2	48.90	±	6.58	4	58.08	±	2.98	0.58	0.03
unknown 4	1231					**2**	**0.05**	**±**	**0.00**	**4**	**0.63**	**±**	**0.11**	0.95	0.001
(*Z*)-β-ocimene[a]	1231	11	0.04	±	0.07	**2**	**0.05**	**±**	**0.01**	**4**	**0.14**	**±**	**0.06**	0.64	0.04
unknown 5	1237	3	0.06	±	0.15										
γ-terpinene[a]	1243	**16**	**0.20**	**±**	**0.12**	2	0.28	±	0.01	4	0.02	±	0.01	0.67	0.02
(*E*)-β-ocimene[a]	1247	15	0.12	±	0.09	**2**	**0.10**	**±**	**0.01**	**4**	**0.72**	**±**	**0.27**	0.62	0.02
p-cymene[a]	1265	16	0.43	±	0.15	2	0.43	±	0.06	4	0.75	±	0.04		
terpinolene[a]	1279	16	0.55	±	0.21	2	0.56	±	0.002	4	1.33	±	0.22		
unknown 6	1332	15	0.15	±	0.06	2	0.14	±	0.01	4	0.40	±	0.08		
p- menthatriene[b]	1385	7	0.01	±	0.02	**1**	**0.03**	**±**	**0.04**	**4**	**0.07**	**±**	**0.01**	0.61	0.02
unknown 7	1422	10	0.04	±	0.04	**2**	**0.09**	**±**	**0.02**	**4**	**0.14**	**±**	**0.02**	0.65	0.02
Alcohols															
thujol	1348					2	0.84	±	0.06						
carvacrol	1354					**2**	**0.84**	**±**	**0.06**	**4**	**0.07**	**±**	**0.02**	1.00	0.001
trans-2,8-menthadienol	1626	**16**	**0.14**	**±**	**0.08**	2	0.12	±	0.01	2	0.09	±	0.11	0.64	0.01
Ethers															
Dill ether[b]	1520	4	0.01	±	0.02					**4**	**0.55**	**±**	**0.18**	0.60	0.01
Epoxides															
(*Z*)-limonene oxide[b]	1443					**1**	**0.02**	**±**	**0.03**	**3**	**0.04**	**±**	**0.04**	0.67	0.001
Sesquiterpenes															
Hydrocarbons															
unknown 8	1465	15	0.07	±	0.05	2	0.07	±	0.02	4	0.14	±	0.02		
unknown 9	1469	16	0.89	±	0.55	2	0.68	±	0.06	4	1.54	±	0.42		
α-copaene[a]	1488					**2**	**0.10**	**±**	**0.05**	**4**	**0.14**	**±**	**0.02**	0.97	0.001
β-elemene[a]	1593	16	0.21	±	0.15	2	0.19	±	0.01	4	0.21	±	0.12		
(*E*)-β-caryophyllene[a]	1606	**16**	**9.09**	**±**	**3.55**	2	6.35	±	0.59	4	10.26	±	2.57	0.58	0.02
unknown 11	1627					**2**	**0.10**	**±**	**0.02**	**2**	**0.01**	**±**	**0.01**	0.67	0.002
γ-elemene[a]	1640	9	0.03	±	0.03	**2**	**0.04**	**±**	**0.01**	**2**	**0.09**	**±**	**0.01**	0.66	0.02
α-humulene[a]	1675	**16**	**1.80**	**±**	**0.82**	2	1.24	±	0.09	4	2.11	±	0.48	0.57	0.02

Table 1 (continued)

Compounds	KI[c]	Aromata N=16				Santa Clara N=2				Carmen N=4				Indicator compounds[e]	
		n[d]	%			n	%			n	%			*IV*	*P*
germacrene D[a]	1713	14	0.12	±	0.08	2	0.11	±	0.02	4	0.17	±	0.10		
δ-cadinene	1764									1	0.01	±	0.02		
germacrene B[b]	1836	6	0.03	±	0.04					4	0.06	±	0.01		
Oxidized Sesquiterpene															
unknown 12	1513	**16**	**0.16**	**±**	**0.05**	2	0.22	±	0.04	4	0.06	±	0.04	0.62	0.01
caryophyllene oxyde[a]	1992	12	0.10	±	0.11	2	0.13	±	0.01	4	0.14	±	0.02		
Benzenoids															
Hydrocarbons															
unknown 13	1166	**16**	**2.01**	**±**	**0.73**	2	0.13	±	0.01	4	0.63	±	0.09	0.76	0.001
unknown 14	1288	**16**	**0.19**	**±**	**0.07**					4	0.41	±	0.09	0.61	0.03
unknown 15	1290	12	0.02	±	0.02					4	0.09	±	0.02		
unknown 16	1296	9	0.02	±	0.02					4	0.08	±	0.02		
unknown 17	1326	11	0.03	±	0.03					4	0.10	±	0.02		
α-p-dimethylstyrene[b]	1431	7	0.01	±	0.02	**2**	**0.03**	**±**	**0.00**	**4**	**0.09**	**±**	**0.01**	0.75	0.002
Unknown															
unknown 18	1377					**1**	**0.01**	**±**	**0.02**	**3**	**0.02**	**±**	**0.02**	0.67	0.001
unknown 19	1705	5	0.02	±	0.03	**2**	**0.04**	**±**	**0.00**	**4**	**0.23**	**±**	**0.09**	0.83	0.001
unknown 20	2056	5	0.01	±	0.02					3	0.04	±	0.03		
unknown 21	2289	2	0.01	±	0.03					**4**	**0.06**	**±**	**0.02**	0.53	0.022

[a] Identification according to retention times and mass spectra in comparison with authentic standards

[b] Identification according to mass spectra and Kovats retention indices (KIs) in comparison with reference libraries and comparison of KIs on similar columns

[c] Kovats retention index

[d] Number of headspace samples in which the compound was present

[e] Indicator compounds are volatiles with a significant observed indicator value (*IV*), which is a measure for the occurrence of volatiles in different samples and/or their relative abundance (*IV*max=1). The deviation of the observed *IV* from a random *IV* was tested using a Monte Carlo test with 10000 randomizations. Bold-faced compounds are characteristic for the respective group, either for Aromata or for Santa Clara/Carmen, according to the *IV*

A choice test (Fig. 2c) confirmed the effect of the cultivar on oviposition ($F_{1,78}$ 32.08, P<0.001). Here again, the females preferred Santa Clara and Carmen over Aromata ($F_{1,78}$=22.96, P<0.001 and $F_{1,78}$=36.11, P<0.001, respectively). In both non-choice and choice tests, there was no difference in the preference between Santa Clara and Carmen (df=1, χ^2=0.56, P=0.79, and $F_{1,78}$=1.53, P=0.22, respectively). Females laid significantly more eggs when in contact with tomato leaves of *S. lycopersicum* cv. Aromata, Carmen and Santa Clara, compared to when they were exposed only to leaf headspace (df=1, χ^2=766.55, P<0.001; Fig. 2).

Comparison Between Cultivated and Wild Tomato Both in the wind tunnel as well as in oviposition bioassays, *T. absoluta* preferred cultivated tomato *S. lycopersicum* over wild tomato *S. habrochaites* ($F_{1,20}$=109.30, P<0.001; $F_{1,72}$=46.42, P<0.001, respectively). The proportion of insects preferring *S. lycopersicum* was similar in both experiments (Fig. 3).

Chemical Analysis of Tomato Headspace We found 52 volatile compounds in the headspace collection of cv. Aromata, Santa Clara, and Carmen (Table 1). Terpenoid compounds largely dominated the tomato leaf headspace. Beta-phellandrene was the most abundant compound by far and represented more than half of the total volatile emission. In addition, limonene, 2-carene, as well as (*E*)-β-caryophyllene were major compounds in tomato headspace of the three cultivars.

Although most volatiles were released by all three cultivars (Table 1), leaf headspace composition differed among them (Permanova $F_{2,19}$=5.95, P<0.001; Fig. 4), headspace differed between Aromata and Santa Clara/Carmen (Permanova

$F_{1,20}$=2.23, P=0.001). According to a Monte Carlo test, 26 of the 52 compounds were good indicators of the leaf headspace of either Aromata or Santa Clara/Carmen (Table 1), most of which are minor terpenoid compounds, with a mean leaf headspace proportion of<1%. Aromata did not produce extra compounds compared to the two other cultivars.

In contrast, Carmen and Santa Clara released 6 compounds that were absent from the headspace of Aromata: carvacrol, α-copaene, (*Z*)-limonene oxide, an unidentified monoterpene (unknown 4), an unidentified sesquiterpene (unknown 11), and one unindentified compound (unknown 18). In addition, 9 other compounds that were characteristic for Santa Clara/Carmen were present in smaller amounts in Aromata leaf headspace. On the other hand, 10 compounds were characteristic for Aromata and more abundant than in Santa Clara and Carmen headspace (Table 1).

Discussion

Combined headspace analysis and behavioral assays show that tomato leaf volatiles are essential cues for host finding and oviposition in tomato leafminer *T. absoluta* (Figs. 1, 2 and 3). The females detected small variations in volatile signatures of the different tomato cultivars (Table 1, Fig. 4), and the ensueing behavioral response (Figs. 2 and 3) matched the suitability of tomato cultivars and species as larval hosts (Leite *et al*., 1999, 2001; Oliveira *et al*., 2009).

Long Range Attraction Cues Mated *T. absoluta* females responded to tomato leaves by upwind oriented flight, followed by landing (Fig. 1). Host volatiles have been shown to elicit attraction of several other moths (Tasin *et al*., 2006; Arab *et al*., 2007; Masante-Roca *et al*., 2007; Knudsen *et al*., 2008; Pinero and Dorn, 2009; Sole *et al*., 2010).

Only mated females were attracted to tomato leaves (Fig. 1). In most herbivorous insects, host-searching behavior is based not only on the perception of suitable olfactory information, but also on the insect's internal state. Physiological changes such as ovarian maturation, egg production, and mating status can influence the response of insect females to host cues (Thompson and Pellmyr, 1991; Yan *et al*., 1999; Masante-Roca *et al*., 2007). For example, mated grapevine moth *Lobesia botrana* or Egyptian cotton leafworm *Spodoptera littoralis* females are attracted to their larval host plants, not unmated females (Masante-Roca *et al*., 2007; Saveer *et al*. unpublished data).

Oviposition Cues Tomato leaf volatiles alone elicited an oviposition response in *T. absoluta* (Fig. 2a). Moreover, ovipositing females discriminated among different tomato cultivars and between cultivated and wild tomato on the basis of volatile cues (Figs. 2 and 3). However, leaf contact significantly increased the number of eggs laid (Fig. 2a and b), and leaf surface morphology and chemistry is accordingly of importance for oviposition. *Tuta absoluta* females lay eggs uniformly on the under- and upper-side of leaves that are covered with trichomes that provide chemical and mechanical stimuli (Torres *et al*., 2001).

Tomato leafminer females lay their eggs directly on leaves (Torres *et al*., 2001). Accordingly, there is no spatial separation between the oviposition site and plant part that releases the chemical signal that elicits host-finding. In contrast, two closely related species from potato, *Tecia solanivora* and *Phthorimaea operculella*, oviposit in the soil close to the plant, rather than on leaves or stems (Fenemore, 1988; Horgan *et al*., 2007; Karlsson *et al*., 2009).

Variation of Volatile Profile Among Tomato Cultivars The volatile profile of tomato leaves of the cultivars Aromata, Santa Clara, and Carmen was dominated by monoterpenes, in particular β-phellandrene (major compound), limonene, and 2-carene, and the sesquiterpene (*E*)-β-caryophyllene (Table 1), which is in accordance with headspace analysis of other tomato cultivars (Buttery *et al*., 1987; Zhang *et al*., 2008). Nonetheless, overall headspace composition of Aromata differed significantly from Santa Clara and Carmen (Fig. 4), due to differences in blend proportions of minor compounds and due to the absence of several compounds, mostly terpenes, in Aromata (Table 1). Our oviposition

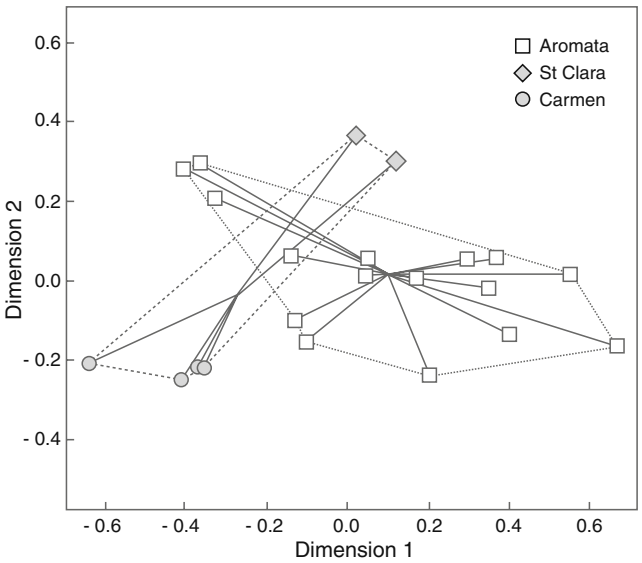


Fig. 4 Non-metric multidimensional scaling (NMDS) plot visualizing the samples of three tomato cultivars, Aromata, Santa Clara, and Carmen based on the similarity (Bray-Curtis distance) of their leaf headspace composition, rotated by principal component, so that the variance of points is maximized on the first dimension (stress=0.15). Samples are grouped (*dashed lines*) according to tomato leafminer *Tuta absoluta* oviposition preference. The centroids of cv. Aromata *vs*. the preferred cvs. Carmen/Santa Clara are the focal points of the *solid lines*

bioassays shows that *T. absoluta* females were able to detect this inter-cultivar variation in leaf headspace, since they laid more eggs in response to headspace from Santa Clara and Carmen than Aromata (Fig. 2).

Tomato Leaf Headspace as an Indicator of Host Suitability Herbivorous moths are attracted to host plant volatiles at specific blend ratios in laboratory assays (Fraser *et al*., 2003; Tasin *et al*., 2006). However, more recent studies in Oriental fruit moth *Cydia molesta* and grapevine moth *Lobesia botrana* show that females are attracted to plant volatile blends that show considerable variation with respect to proportions and composition (Tasin *et al*., 2007, 2010; Najar-Rodriguez *et al*. 2010). A response to a broad spectrum of plant volatile blends could represent an adaptation to differences in volatile signatures from genotypes and to variation during phenological changes and would accordingly facilitate host finding even in specialist herbivores.

This is contrasted by the differential response of tomato leafminer to comparatively small variations in tomato headspace components and proportions (Table 1, Fig. 2). A tentative explanation for discrimination of minor blend variations is that the females are less tolerant and respond strongly to those compounds that are direct cues of host plant suitability. Trichome-borne terpenoids, which account for much of the difference between the cultivars Aromata, Santa Clara, and Carmen (Table 1) have been suggested to influence host plant selection and fitness in tomato insects (Bleeker *et al*., 2009, 2011; Kang *et al*., 2010).

The idea that volatile cues provide information of host suitability at a distance is further substantiated by attraction and egg-laying choice tests with wild tomato *S. habrochaites*, where females clearly preferred cultivated over wild tomato (Fig. 3). Leaf headspace of wild tomato *S. habrochaites* was dominated by β-ocimene, β-myrcene, and undecan-2-one (Smith *et al*., 1996; Bleeker *et al*., 2009). Glandular trichomes of wild tomato also contain many sesquiterpenes, including caryophyllene, curcumene, elemene, humulene, and zingiberene, that play a role in tomato resistance against beet armyworm and whitefly (Eigenbrode *et al*., 1994; Smith *et al*., 1996; van der Hoeven *et al*., 2000; Bleeker *et al*., 2009, 2011). Resistance of wild tomato to tomato leafminer has been attributed to tridecan-2-one, undecan-2-one, and zingiberene, which have not been found in the three cultivars studied here (Leite *et al*., 1999; Azevedo *et al*., 2003).

This initial study of the attraction and oviposition response of tomato leafminer *T. absoluta* in response to its primary host tomato will support the identification of the odor template that encodes host recognition, and the development of new methods to control this important tomato pest worldwide. Tomato volatiles can be used directly for trapping mated females, and the differential behavioral response to tomato cultivars indicates the potential of plant breeding for improved resistance. While a complete description of the bioactive compounds is a considerable undertaking, the wind tunnel bioassay is already available for efficient screening of plant genotypes.

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Synergistic or Antagonistic Modulation of Oviposition Response of Two Swallowtail Butterflies, *Papilio maackii* and *P. protenor*, to *Phellodendron amurense* by Its Constitutive Prenylated Flavonoid, Phellamurin

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Abstract *Papilio maackii* females prefer a rutaceous plant, *Phellodendron amurense*, for oviposition, whereas another semi-sympatric Rutaceae feeder, *Papilio protenor*, never exploits this plant as a host in nature. However, the larvae of both species perform well on this plant in the laboratory. Phellamurin, a flavonoid present in the organic fraction from *P. amurense* inhibits egg laying by *P. protenor*. We examined whether phellamurin is involved in the differential acceptance of *P. amurense* by the two butterflies. The ovipositing females of *P. maackii* readily accepted *P. amurense* and a methanolic extract of the foliage, while *P. protenor* rejected them entirely. However, the aqueous fraction derived from the extract elicited significant oviposition responses of similar levels from the two species. Phellamurin did not induce oviposition behavior in *P. protenor* females. In contrast, *P. maackii* was stimulated to oviposit by phellamurin at concentrations exceeding 0.2%. The response was dose-dependent and reached ca. 70% at 2% phellamurin, which is approximately equivalent to its natural abundance in young leaves of *P. amurense*. Since the aqueous fraction was very stimulatory to both species, the combined effect of phellamurin and the aqueous fraction on oviposition was tested. The addition of phellamurin to the aqueous fraction enhanced the ovipositional activity of *P. maackii*, but dramatically suppressed the oviposition response of *P. protenor* even at 0.1% concentration. These results, taken together with those obtained from electrophysiological recordings with foretarsal chemosensilla, indicate that phellamurin acts as an oviposition stimulant for *P. maackii*, and as a potent deterrent for *P. protenor*. The results suggest that host range expansion or host shifts may be made by ovipositing females that overcome phytochemical barriers.

Key Words Host selection · Host shift · Rutaceae · Dihydroflavonol glucoside · Oviposition stimulant · Oviposition deterrent · Lepidoptera · Papilionidae

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Introduction

Plant secondary metabolites can be exploited by phytophagous insects during host selection. In general, some compounds serve as stimulants/attractants for feeding or oviposition, while others act as deterrents/repellents (e.g., Schoonhoven et al., 2005; Honda et al., 2010). Although the host range or host-preference hierarchy of an insect is regionally variable, the majority of herbivorous insects use only a limited range of plant species, mostly of a single or few families, or only one plant species at the extreme. Such host specificity may be due to a species-specific capacity for metabolizing allelochemicals (Berenbaum, 1991; Feeny, 1991) and/or limitations on processing information in the central nervous system (Bernays, 2001). Extensive work on chemical mediators in oviposition by lepidopterans has demonstrated clearly that chemical attributes of plants serve as the crucial cues that permit females to discriminate and recognize their hosts (Renwick and Chew, 1994; Honda, 1995, 2005; Honda et al., 2010).

In butterflies, assessment of potential hosts is achieved mainly by drumming on the surface of foliage with foretarsi endowed with chemotactile sensilla that can perceive plant chemicals (Inoue, 2006). Acceptance or rejection of a given plant by ovipositing females is governed not only by the presence or absence of stimulatory substances, but also by other co-occurring constituents that exert inhibitory activities, hence, by a reciprocal balance of positive and negative sensory inputs received upon contact with the plant (Renwick and Chew, 1994; Honda, 1995).

While host-plant affiliations for papilionid butterflies include many plant families, 80% of butterflies in the genus *Papilio* feed on rutaceous plants. A wide array of phytochemical stimulants and deterrents responsible for oviposition has been reported for *Papilio* butterflies that feed on Rutaceae, Apiaceae, or Lauraceae (Honda, 1995; Honda et al., 2010). Major compounds identified include amino acid derivatives, sugar-related acids, alkaloids, flavonoids, and hydroxycinnamic acid derivatives. Among the stimulants for *Papilio* butterflies, some are common stimulants for other species, and others are structurally related to one another, thereby strongly suggesting the involvement of phytochemicals in host specialization on one hand, and expansion of host range on the other (Feeny, 1991). Many investigations have been conducted on assumed host shifts of papilionids, presumably originating from Aristolochiaceae, via Lauraceae, Magnoliaceae, Annonaceae, and Rutaceae, eventually leading to Apiaceae and Asteraceae (e.g., Aubert et al., 1999; Scriber et al., 2007, 2008).

In this study, we focused attention on two Rutaceae specialists, *Papilio maackii* and *P. protenor*, that have quite different host ranges in order to clarify the phytochemical and electrophysiological bases for the differential acceptance of *Phellodendron amurense* by ovipositing females. The two butterfly species range from temperate to boreal regions of Japan, with broad overlapping habitats on the main island (Honshu). *Papilio maackii* is rather montane, and prefers cool climates, while *P. protenor* is common in warm districts but relatively rare in montane or northern districts. Therefore, their occurrence is somewhat allopatric in most temperate regions. *Papilio maackii* utilizes *P. amurense* as a primary host plant in almost all habitats but *P. prontenor* females completely reject this species although the larvae perform well on this plant in the laboratory (Honda and Hayashi, 1995a). By contrast, *Papilio protenor* exclusively utilizes *Citrus* plants throughout its habitats.

Phellamurin [3,5,7,4′-tetrahydroxy-8-(3-methylbut-2-enyl)flavanone-7-*O*-ß-glucoside], which is present in *P. amurense*, strongly deters oviposition in a Kanagawa population of *P. protenor demetrius* (Honda and Hayashi, 1995b). Although many studies have investigated oviposition stimulants for *Papilio* butterflies, less attention has been paid to ecologically significant oviposition deterrents. It is important to understand how inhibitory phytochemicals may have played a role in establishing present-day host use and host-range evolution. The aim of this study was to examine how *P. maackii* females respond to phellamurin in their ovipositional behavior, and how the compound stimulates the foretarsal chemosensory receptors in both *P. maackii* and *P. protenor*. We also evaluated the oviposition response to *P. amurense* and phellamurin in a population of *P. protenor demetrius* from Hiroshima for comparison to previous work on a population from Kanagawa.

Methods and Materials

Insects Larvae of *Papilio maackii* and *P. protenor demetrius* were reared at 25°C under a photoregime of 16:8 hL:D on potted plants of *Phellodendron amurense* and *Citrus* spp., respectively. These were laboratory stock cultures that originated from females collected in Hiroshima Prefecture. Adults of both species used for the behavioral bioassays were 3- to 10-d-old gravid females, which had been hand-paired or allowed to copulate in an outdoor cage (7×10 m, 3.5 m high). Females were hand-fed with 15% aqueous sucrose solution once daily throughout the experiments.

Extraction and Fractionation of Plant Materials Young leaves of *P. amurense* (600 g) were extracted with methanol (4 l) at room temperature for 1 mo. The methanolic extract was concentrated *in vacuo* below 50°C, and an aliquot of the whole concentrate, after being dispersed in water, was successively partitioned with chloroform and isobutanol to give 3 fractions (1 aqueous and 2 organic). The chloroform-soluble fraction (Fr. 1) and isobutanol-soluble fraction (Fr. 2) were evaporated to dryness below 50°C, and each residue was re-dissolved in chloroform and in methanol, respectively. The water-soluble fraction (Fr. 3) was lyophilized and dissolved in 50% aqueous methanol. All fractions were stored below 0°C until use. Phellamurin was isolated from Fr. 2 (Honda and Hayashi, 1995b).

Bioassay for Oviposition Response Behavioral bioassays were conducted by a method similar to that reported previously (Honda, 1990), employing a green heart-shaped plastic plate (2–5 cm^2) as a leaf surrogate. Before testing, females were screened daily to assess their responsiveness, and only those that exhibited positive responses to the foliage of *P. amurense* (for *P. maackii*) or *Citrus natsudaidai* (for *P. protenor*) and negative responses to water alone (control) were chosen. In each trial (sample presentation), the response of an individual was scored as 100% for actual egg laying or an equivalent behavior

(trying to bring the ovipositor in contact with the underside of the leaf without egg deposition), 50% for half-curling the abdomen with continuous drumming (this behavior took place infrequently), and 0% for drumming only, with no attempt to oviposit (Nakayama et al., 2002). Trials were replicated at least three times for each individual, and the responses of an individual to a given sample were averaged. For all trials, merely alighting on ovipositional substrates without drumming was not counted. Oviposition response to each sample was eventually represented as the mean percentage of responses recorded from at least 10 females.

Concentrations of test solutions are all expressed as % w/v. The amount of dry test materials on the artificial leaf was estimated. For example, if a 0.5% solution of test sample was applied to the leaf, the amount of material was estimated at 50 μg/cm^2. Test samples prepared from the initial extract, the three partitioned fractions (Frs. 1 to 3), and phellamurin were tested at several concentrations for their stimulatory activity in ovipositioning. For test samples of a binary mixture, Fr.3 (0.5%) was used as a base component, since it exhibited potent oviposition-stimulatory activity (more than 90% response) for both species.

Electrophysiological Recordings The 5th tarsomere of the female foretarsi of both species possesses bristles with a large number of long (major) and short sensilla trichodea (Ryan, 2002) that are situated between 2 lateral and a midline row of spines. The foretarsi also bear a small number of similar lateral sensilla. The responses of the foretarsal chemosensilla to phellamurin, Fr. 3, and their mixtures were examined electrophysiologically. To record responses from the sensilla, tip recording was performed using an ER-1 extracellular amplifier (Cygnus Technology, Inc., USA) and a PowerLab 4/25 converter (ADInstruments Pty Ltd., Australia). Data sampling was conducted at the rate of 10 k/s (low pass filter: 3 kHz; high pass filter: 100 Hz; and sampling gain: 100). Forelegs were amputated at the base of the tibia and fixed on the stage with double-faced adhesive tape. A sharpened tungsten needle (indifferent electrode) was inserted into the first tarsomere. Under a binocular microscope, a glass capillary tube (recording electrode) filled with a test solution was brought into contact with the tip of a medial long trichoid sensillum of the 5th tarsomere. Successive stimulations of a sensillum were of 1 s duration and were separated by an interval of at least 3 min. One to 5 sensilla of the same type were randomly selected from the same preparation. A total of 18 sensilla from 6 females of *P. maackii* and 19 sensilla from 7 females of *P. protenor* were subjected to the measurement. Spike trains were analyzed with Spike Histogram software (ADInstruments Pty Ltd., Australia). The frequencies (F) and average intensities (I) of spikes with amplitudes higher than 10 μV were quantified, regardless of their spike types, using 5 recordings for each stimulus. Test materials (stimuli) were dissolved in 6–12% aqueous ethanol containing 10 mM NaCl as an electrolyte.

Results

Oviposition Responses to the Foliage, Extract, and Fractions of Phellondendron amurense Ovipositing *P. maackii* females responded strongly to the foliage, methanol extract, and Fr. 3, and moderately to Fr. 2., while Fr. 1 (a mixture of less polar substances) was weakly active (Fig. 1). It was noteworthy that *P. maackii* females responded positively to Fr. 2, as this isobutanol-soluble fraction has been reported to be almost inactive by itself for the Rutaceae-specialized *Papilio* species thus far investigated (Honda and Hayashi, 1995a; Nakayama et al., 2002; Murakami et al., 2003; Chachin et al., 2007).

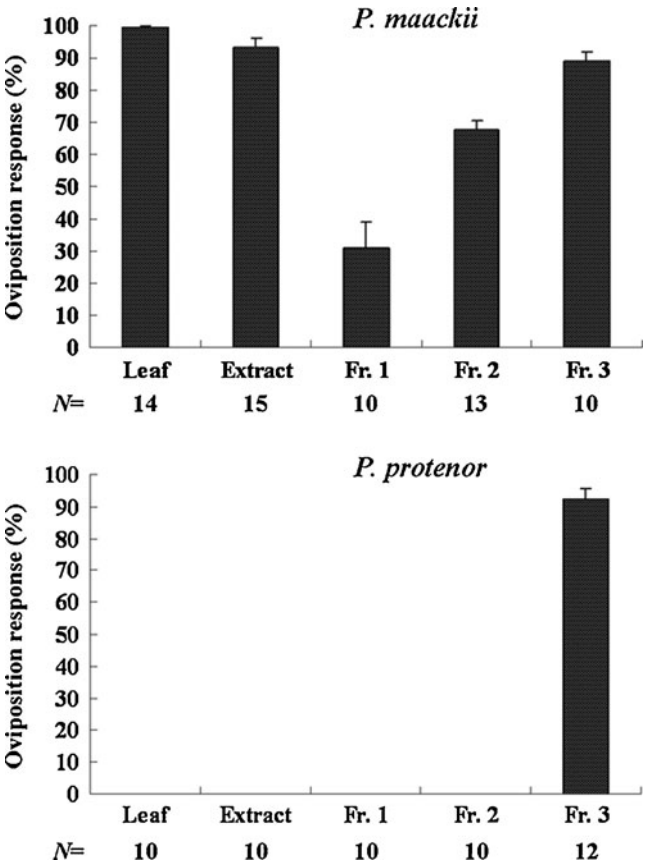


Fig. 1 Oviposition responses (mean±SE) of the two species (*Papilio maackii* and *Papilio protenor*) to the foliage of *Phellodendron amurense*, the methanol extract of the foliage, and fractions of the extract prepared as described in the text. The extract and fractions were tested at 0.5%

In marked contrast, *P. protenor* females responded positively only to Fr. 3, and totally rejected the other fractions (Fig. 1). The response of *P. protenor* females of the Hiroshima population to each sample was virtually identical to that of the Kanagawa population reported previously (Honda and Hayashi, 1995a, b).

Oviposition Response to Phellamurin Females of *P. maackii* had positive oviposition responses to phellamurin in a dose-dependent manner (Fig. 2). With 2.0% phellamurin, corresponding to the concentration of the compound found in young leaves of *P. amurense* (Honda and Hayashi, 1995b), the response was 72.4%. Ovipositing females of *P. protenor* did not respond to phellamurin.

Mixtures of Phellamurin and Fr. 3 As shown in Fig. 3, the addition of phellamurin enhanced oviposition responses of *P. maackii* females to Fr. 3. In *P. protenor*, the stimulatory activity of Fr. 3 was suppressed upon addition of a very small dose (0.1%) of phellamurin. There was almost complete rejection at higher doses (0.2% or more). This tendency is similar to that reported previously for the Kanagawa population (Honda and Hayashi, 1995b), though individuals of the Hiroshima population appear to be more sensitive to phellamurin.

Electrophysiological Responses Recordings from foretarsal taste sensilla were conducted with aqueous solutions of ethanol (solvent), phellamurin, Fr. 3, and their mixtures. Sucrose was used as the reference stimulus. Figure 4 shows typical recording traces for *P. maackii* and *P. protenor*. The sensilla of both species were unresponsive to the solvent

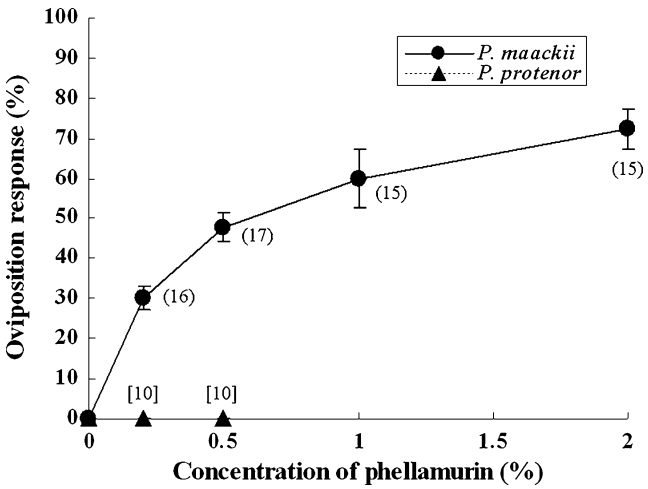


Fig. 2 Oviposition responses (mean±SE) of the two species (*Papilio maackii* and *Papilio protenor*) to various concentrations of phellamurin . The number of individuals tested is given in parentheses or brackets

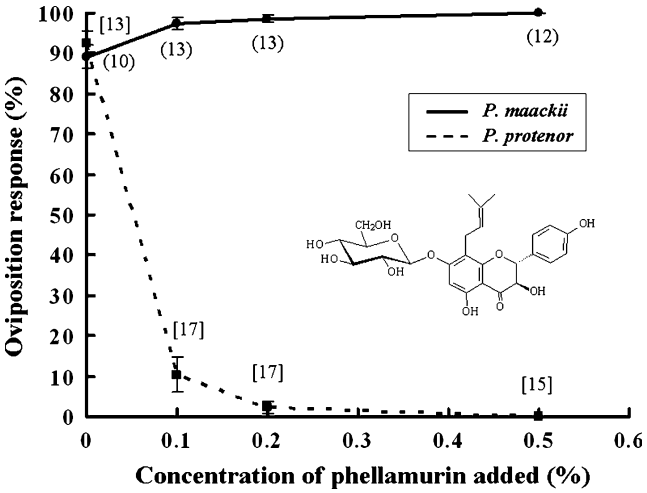


Fig. 3 Dose-response relationships for phellamurin mixed with Fr. 3. The oviposition behavior of the two species (*Papilio maackii* and *Papilio protenor*) expressed as mean±SE for various concentrations of phellamurin mixed with Fr. 3 (0.5%). The number of individuals tested is given in parentheses or brackets

(12% ethanol) and 62.5 mM sucrose (traces 1 and 6), but exhibited unambiguous responses to stimulation with the other solutions. The sensilla of *P. maackii* were more intensely stimulated with phellamurin and/or Fr. 3 than were those of *P. protenor*, with the former displaying spikes of lower amplitude. In both species, phellamurin appeared to evoke regular spikes from 1 neuron (trace 2). The frequencies (F; mean±SD) and intensities (μV) (I; mean±SE) of spikes evoked in the sensilla of *P. maackii* and *P. protenor* were (F) 39±19, (I) 210±14, and (F) 29±9, (I) 341±15, respectively.

In contrast, Fr. 3 , which contained less than 62.5 mM sucrose, elicited several types of spikes with different amplitudes, and its stimulation mode for *P. maackii* was diverse as compared to that for *P. protenor* (trace 3). The spike trains of both species were so complicated that we were only able to approximately evaluate their response patterns by lumping together all spike types. The mean spike frequencies and intensities of *P. maackii* and *P. protenor* were (F) 102±9, (I) 312±19, and (F) 26±6, (I) 407±20, respectively.

Although the spike trains of *P. maackii* in response to the mixtures of phellamurin and Fr. 3 (traces 4 and 5) were too complicated to analyze with the software available, the mean spike frequency and intensity [(F) 104±4 and (I) 271±27] recorded by stimulation with 0.5% Fr. 3 plus 0.2% phellamurin (trace 5) were similar to those observed with Fr. 3 alone. The sensory response pattern in *P. protenor* to the same mixture (trace 5) was less complex. The impulses evoked by stimulation with mixture [(F) 68±20 and (I) 426±21] may have represented an additive response because the

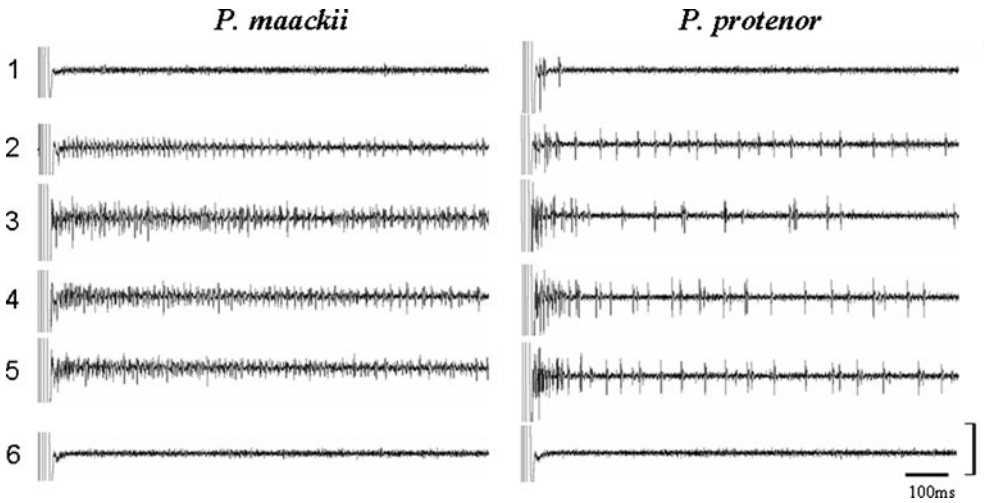


Fig. 4 Electrophysiological recordings from the foretarsal medial large sensilla (s. trichodea) for the two species (*Papilio maackii* and *Papilio protenor*). Examples of traces in response to aqueous solutions of (1) 12% ethanol, (2) 0.2% phellamurin +12% ethanol, (3) 0.5% Fr. 3+10% ethanol, (4) 0.5% Fr. 3+0.1% phellamurin +6% ethanol, (5) 0.5% Fr. 3+0.2% phellamurin +12% ethanol, and (6) 62.5 mM sucrose. Each sample contained NaCl (10 mM) as an electrolyte and all traces were from the same sensillum. Amplitude scale bar: 500 μV

spike count of the mixture was virtually equivalent to the sum total of spikes evoked by the respective samples.

Discussion

The rejection of *Phellodendron amurense* by ovipositing females of *Papilio protenor* can be ascribed to negative effects of phellamurin in the foliage. Fraction 1 had no oviposition-inhibitory effect (Honda and Hayashi, 1995a). The mixture we tested (Fig. 3) composed of 0.5% Fr. 3 and 0.25% phellamurin, approximated the relative concentrations in the living plants., We conclude that the deterrent activity of phellamurin far outweighs the potent stimuli evoked by water-soluble substances (Fr. 3), thus resulting in persistent avoidance of the plant by females. The essential agreement on the dose-response relationship for phellamurin between Kanagawa and Hiroshima populations (located 650 km apart) indicates that the circumvention of phellamurin by *P. protenor* females is not a regionally-expressed but species-specific trait.

Fraction 2 (isobutanol-soluble substances) consists mainly of flavonoid glycosides, including phellamurin as a major component (Honda and Hayashi, 1995b). Thus, phellamurin is most likely responsible for the positive response to Fr. 2 by *P. maackii* females. Phellamurin alone exerted considerable oviposition-stimulatory activity for *P. maackii*, in contrast to other flavonoid stimulants that are effective only when they operate together with other structurally unrelated substances (Ohsugi et al., 1985; Nishida et al., 1987; Honda, 1990). Furthermore, marked synergism of multiple components in host recognition generally prevails in *Papilio* butterflies (Renwick and Chew, 1994; Honda, 1995; Honda et al., 2010). In addition to its direct action in stimulation of oviposition, phellamurin synergistically enhanced the responses of *P. maackii* females to Fr. 3 (Fig. 3). Thus, for *P. maackii*, phellamurin acts as an independent or a synergistic oviposition stimulant probably involved in host preference.

The foretarsal sensilla of the two butterfly species exhibited species-specific electrophysiological responses to Fr. 3 and phellamurin. In both species, the spikes generated by stimulation with Fr. 3 (trace 3) seem to originate from at least 2 or more different sensory receptor cells that are responsive to some as yet uncharacterized oviposition stimulants present in the fraction. Despite an enormous difference in its effect on the two butterflies' behavior, phellamurin elicited one type of spikes from both species. In *P. protenor*, phellamurin (a deterrent), which is received by a specific cell distinct from those involved in the reception of stimulant(s), would transmit a behaviorally negative signal to the central nervous system. In *P. maackii*, however, phellamurin might stimulate one of the neurons that are responsive to components in Fr. 3.

Electrophysiological experiments have been carried out for some butterfly species in relation to oviposition behavior (e.g., Roessingh et al., 1991; Du et al., 1995; Städler et al., 1995). Investigations of 2 pierids, *Pieris napi* and *P. rapae*, revealed that one of the taste neurons responsive to glucosinolates (oviposition stimulants) is also sensitive to cardenolides (oviposition deterrents), and further have provided evidence for peripheral interactions of stimulant compounds with a neuron responding to deterrents (Du et al., 1995; Städler et al., 1995). Other instances of interference with chemoreceptor activity caused by inhibitory chemicals have been reported for other lepidopterans (e.g., Schoonhoven and Fu-Shun, 1989; van Loon, 1996; Qiu et al., 1998). None of the recordings we observed, however, found clear indications of such peripheral interaction of phellamurin with Fr. 3 sensitive cells (trace 5). Therefore, sensory events at the periphery,

unlike an interference mechanism, seem likely to underlie the rejection of phellamurin-containing plants by ovipositing *P. protenor* females.

The genus *Citrus* is one of the plant taxa exploited worldwide by Rutaceae-feeding *Papilio* butterflies. Phytochemical data compiled to date show that flavonoid compounds abundant in *Citrus* species are flavones, flavanones, and flavonols, while compounds like phellamurin, which is a *C*-prenyl substituted 2,3-dihydroflavonols (flavanonols), are rarely found in *Citrus* (Tripoli et al., 2007; Ribeiro et al., 2008; Abad-García et al., 2009). *Citrus* plants are not sympatric with *P. amurense* at most locations in Japan; *Citrus* plants generally lack cold hardiness, and thus grow along coastal areas in temperate to warm regions. *Phellodendron amurense* is distributed in lowlands in cold latitudes but usually occurs at higher elevations in temperate regions. Therefore, it seems likely that not only *P. protenor* but other *Citrus* feeders, including *P. xuthus* (Honda and Hayashi, 1995b), have had little chance in their evolutionary history to encounter *P. amurense*. It is surprising that the larvae are able to ingest phellamurin given the female aversion (Honda and Hayashi, 1995a). The genetic grounds for such differential adaptation of adults and larvae to a given plant have been studied in other *Papilio* butterflies (e.g., Thompson et al., 1990).

Some flavonoids are known as oviposition stimulants for some papilionid butterflies (Ohsugi et al., 1985; Nishida et al., 1987; Honda, 1995), whereas flavonoids present in non-host plants often play a significant inhibitory role in regulation of oviposition (Nishida et al., 1990). We previously proposed that the ancestors of Papilionidae might have most heavily relied in host recognition on simple phytochemicals such as amino acid derivatives, hydroxycarboxylic acids, and/or polyols that are closer to primary metabolites (Nakayama et al., 2003). By contrast, flavonoids are relatively advanced secondary metabolites (Harborne and Baxter, 1993). In this context, we presume that flavonoids serve as modulators that govern host choice by ovipositing female, and that positive responses to flavonoids may be an apomorphic trait in the Papilionidae.

According to mitochondrial DNA phylogeny of the Papilionidae (Yagi et al., 1999; Zakharov et al., 2004), Japanese swallowtails in the tribe Papilionini comprise at least 2 subclusters; the subgenus *Menelaides* that includes typical *Citrus* feeders such as *Papilio protenor* and *P. memnon*, and the subgenus *Achillides* that includes *Papilio maackii* and *P. bianor*, both of which utilize *Phellodendron amurense* as a natural host. *Papilio protenor* and *P. maackii* may have diverged from a common ancestor, eventually exploiting distinct host plants and establishing their respective niches. It is noteworthy that other *Menelaides* members, *Papilio polytes* and *Papilio helenus*, also can complete their immature stages on *P. amurense*, and its aqueous fraction exerts strong to weak oviposition-stimulatory activity to *P. xuthus* and *P. polytes* (Honda and Hayashi, 1995a; Nakayama et al., 2002). *Papilio maackii* prefers to live in cool and montane zones, where rutaceous plants available for larval food are restricted to *P. amurense* only, particularly in northern Japan. In light of the present-day species richness of Papilionidae in tropical and temperate regions (Scriber, 1995), it is hypothesized that female chemosensory adaptation of the ancestors of *P. maackii* to an unusual (prenylated) flavonoid might have enabled them to colonize *P. amurense* and to extend their habitat further to boreal regions (as far as ca. 53°N latitude). In addition, *P. bianor*, another member of *Achillides*, has been observed in the field to lay eggs on *Citrus* plants on rare occasions and can marginally survive on them up to adulthood (Endo and Nihira, 1990). Positive oviposition responses to ancestral host plants also have been reported for species of the *Papilio glaucus* group (Mercader and Scriber, 2008). These and our finding that *P. protenor* females are very susceptible to phellamurin also seem to lend support to a possible host shift from *Citrus* plants to *P. amurense* by the common ancestor of *Menelaides* and *Achillides*.

The present finding that the stimulus evoked by a single phytochemical is interpreted differently by related butterfly species, suggests the involvement of chemoreceptor mutation(s). This may afford an opportunity for investigating the sensory and evolutionary mechanisms of chemical mediation of host range expansion and host shifts in the Papilionidae.

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Attractiveness of Constitutive and Herbivore-Induced Sesquiterpene Blends of Maize to the Parasitic Wasp *Cotesia marginiventris* (Cresson)

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Abstract Plant volatile compounds induced by herbivore attack have been demonstrated to provide a signal to herbivore enemies such as parasitic wasps that use these volatiles to locate their hosts. However, in addition to herbivore-induced volatiles, plants often release volatiles constitutively. We assessed the interaction between herbivore-induced and constitutively released volatiles of maize in the attraction of the wasp *Cotesia marginiventris* that parasitizes herbivorous lepidopteran larvae feeding on maize. Experiments were carried out with olfactometers in which the sources of volatiles were transgenic *Arabidopsis thaliana* plants overexpressing maize sesquiterpene synthases that produce blends of herbivore-induced or constitutive compounds. We found that the constitutive volatiles of maize terpene synthase 8 (TPS8) were attractive to *C. marginiventris*, just like the herbivore-induced volatiles of TPS10 studied earlier. A mixture of both the TPS8 and TPS10 volatile blends, however, was more effective in parasitoid attraction, indicating that constitutively released sesquiterpenes enhance the attraction of those induced by herbivores. While *C. marginiventris* did not distinguish among the volatiles of TPS8, TPS10, nor those of another maize sesquiterpene synthase (TPS5), when these blends were combined, their attractiveness to the wasp appeared to increase with the complexity of the blend.

Key Words *Cotesia marginiventris* · *Arabidopsis thaliana* · *Zea mays* · *Spodoptera littoralis* · Sesquiterpenes · Volatile organic compounds (VOCs) · Herbivore-induced volatiles · Constitutive volatiles · Plant defense · Parasitoid attraction

Introduction

Plants emit a plethora of volatile organic compounds (VOCs) from their vegetative organs into the surrounding atmosphere. The most common are green leaf volatiles (GLVs, C_6 alcohols, aldehydes, and derivatives), and terpenes, especially monoterpenes and sesquiterpenes (Dudareva et al., 2004). VOCs are released abundantly after herbivore attack, but undamaged plants, especially tree species, also show high emission rates (Kesselmeier and Staudt, 1999). For example, undamaged poplars (*Populus* spp.) can invest more than 10% of their photosynthetically fixed carbon into the emission of isoprene (Brilli et al., 2009). The majority of studies on vegetative VOCs have focused on herbivore-induced volatiles and provided ample evidence for their role

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in attraction of herbivore enemies. These studies include agronomically important crops (e.g., maize, tomato, soybean, cabbage) as well as wild species, both herbaceous and woody (reviewed by Unsicker et al., 2009). Much less is known about the role of constitutively emitted volatiles in plant indirect defense, as these rarely have been studied separately from the herbivore-induced ones. Attraction of herbivore enemies to uninfested plants has been documented for some aphid parasitoids (Hymenoptera: Braconidae) (reviewed by Hatano et al., 2008) and for two lepidopteran parasitoids (Elzen et al., 1986, 1987).

Herbivore-induced and constitutive VOCs often are released in complex blends (Dicke et al., 2009). Since these natural mixtures are difficult to manipulate or to reproduce synthetically, most studies have tested arthropod attraction to either whole blends, or to single VOCs available in pure form. For example, the GLV (*E*)-3-hexenol is attractive to the braconid wasp *Apanteles* (=*Cotesia*) *kariyai* (Watanabe) (Takabayashi et al., 1991), and the sesquiterpenes γ-bisabolene and (*E*)-β-caryophyllene attract *C. sonorensis* females. These ichneumonids oviposit in noctuid larvae that feed on cotton plants, whose vegetative parts are rich in sesquiterpenes (reviewed by Rutledge, 1996). Single volatiles responsible for the attraction of herbivore enemies also have been identified in a few aphid-parasitoid systems (reviewed by Hatano et al., 2008) and in one mite - predatory mite system (de Boer and Dicke, 2004; de Boer et al., 2004). However, there are cases in which the whole volatile blend released by a plant is necessary for the parasitoid to locate its host's habitat. A study with the egg parasitoid *Closterocerus ruforum* (Krausse) (Hymenoptera: Eulophidae) demonstrated that the whole VOC blend emitted by Scots pine (*Pinus sylvestris* L.) after oviposition by the herbivorous sawfly *Diprion pini* (L.) attracts the parasitoid (Mumm and Hilker, 2005). This blend differs from that of uninfested plants by having greater amounts of (*E*)-β-farnesene. However, neither (*E*)-β-farnesene, nor the VOC blend of uninfested plants by itself was attractive to the parasitoids; both were required.

Maize (*Zea mays* L.) is one of the best studied species for the role of volatiles in indirect plant defense. The volatile bouquet emitted by seedlings after feeding by generalist lepidopteran caterpillars (*Spodoptera littoralis* (Boisd.), *S. exigua* (Hübn.)) has been well characterized and studied for over two decades (D'Alessandro et al., 2009; Turlings et al., 1990). Important components of this odor are GLVs (esters and aldehydes) with their abundance dependent on the extent and intensity of herbivore damage. The monoterpene alcohol linalool, the C_{11} homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and indole also are present often in high amounts. However, the biggest proportion of the blend is made up of (*E*)-β-farnesene, (*E*)-α-bergamotene, and other sesquiterpene hydrocarbons (Turlings and Ton, 2006). This herbivore-induced blend is attractive to parasitic wasps (Turlings et al., 1990, 1991), raising the question of whether the entire mixture or individual components are responsible for the attraction. In order to tackle this question, the volatile mixture was fractionated (D'Alessandro and Turlings, 2005), and plants have been engineered to emit only a specific fraction of the terpene blend. Transgenic *Arabidopsis thaliana* plants emitting the volatiles formed by maize terpene synthase 10 (TPS10), which make up most of the herbivore-induced sesquiterpenes of maize leaves, were more attractive than untransformed controls to the parasitoid wasp *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) in olfactometer experiments (Schnee et al., 2006).

In addition to herbivore-induced terpenes, many lines of maize emit an additional sesquiterpene mixture, which is constitutively emitted by the roots and aboveground vegetative tissues of young plants. These terpenes are synthesized by TPS8 (Fig. 1a), a terpene synthase that is expressed independently of herbivory in the inbred line B73 as well as in several other cultivars and wild ancestors of maize (Köllner et al., 2004b, 2008, 2009). The ecological role of maize TPS8 products has been investigated in the context of plant-pathogen interactions and deterrence of insect herbivory, and these substances have been shown to act as defenses against fungi, but not against herbivores (Fontana et al. unpublished data). The terpene blend produced by TPS8 is extraordinarily complex,and one major component of this mixture, (*E*)-β-caryophyllene, has been implicated in the attraction of herbivore enemies both above and below ground (Köllner et al., 2008). A third sesquiterpene blend dominated by sesquithujene and β-bisabolene is produced by TPS5 in the husks and at lower levels in the leaves of mature maize plants (Köllner et al., 2004a). As young instars of generalist lepidopteran larvae are not normally found on these plant parts, the TPS5 blend is unlikely to be used by their parasitoids for host location.

Here, we studied the function of the constitutively emitted TPS8 terpenes in indirect plant defense. The study system involved the generalist parasitic wasp *C. marginiventris,* which oviposits on lepidopteran larvae feeding on maize. The constitutively emitted TPS8 volatiles may act: 1) together with the herbivore-induced (TPS10) terpenes to strengthen attraction of the wasps; 2) antagonistically with the herbivore-induced signal to weaken the parasitoid attraction; or 3) without effect, neither enhancing nor diminishing the parasitoid response to the herbivore-induced odor. To test these hypotheses, we performed a series of olfactometer experiments where we measured the attraction of *C. marginiventris* females to the TPS10 or TPS8 terpene blends, either singly or in combination, as emitted from transgenic *A. thaliana* lines. We also tested whether the

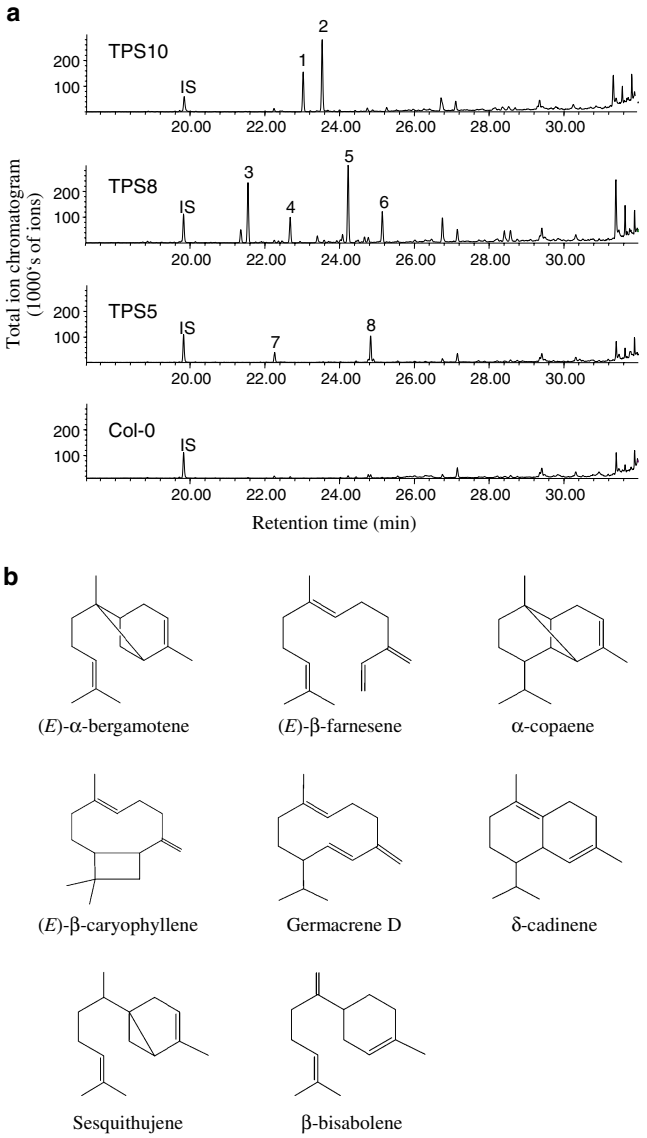


Fig. 1 **a** Volatile profile of *Arabidopsis thaliana* rosette stage plants transformed with *tps10*, *tps8*, and *tps5*, and of untransformed Col-0 plants. The main volatile compounds were identified as: 1. (*E*)-α-bergamotene; 2. (*E*)-β-farnesene; 3. α –copaene; 4. (*E*)-β-caryophyllene; 5. germacrene D; 6. δ-cadinene; 7. sesquithujene; 8. β-bisabolene. Their structures are shown in **b**. IS: internal standard

parasitoid's responses to the terpene blends are innate or learnt by association with an oviposition experience. It has been shown that *C. marginiventris* females can be attracted to different odors (among which is the odor of herbivore-induced maize) only after they had associated them to an oviposition experience (Hoballah and Turlings, 2005; Schnee et al., 2006). However, it is not known whether the parasitoids learn the whole blend or only some of its components, nor how specifically they learn the individual constituents. Finally, by using a third maize terpene blend, that of TPS5, we investigated whether the complexity of the mixture is an important variable in parasitoid attraction.

Methods and Materials

Generation of Transgenic Arabidopsis thaliana *Plants.* The generation of transgenic TPS10 (Schnee et al., 2006) and TPS8 (Fontana et al. unpublished data) plants has been described elsewhere. For TPS5 plants, the open reading frame (ORF) of *tps5* from *Zea mays* var. Delprim (*tps5-Del1*) was amplified from the pASK-IBA7 construct (Köllner et al., 2004b) with the primers forward (*tps5*fwd): 5′-ATGGCGTCTCCTCCAGCACATCG-3′ and reverse (*tps5*rvs): 5′-TCATTCGGGTATTGGCTCCACAAACAG-3′ and cloned into the pCR-TOPO vector (Invitrogen, Carlsbad, CA, USA) for sequencing. After sequence analysis, the 1665 bp ORF was re-amplified from the sequencing vector and cloned using Gateway technology (Karimi et al., 2002) into the plant expression vector pB2GW7 between the p35S promoter and the T35S terminator of the cauliflower mosaic virus. The resulting construct was introduced into the *Agrobacterium tumefaciens* strain GV3101, which was then used to transform *Arabidopsis thaliana* L. (ecotype Col-0) plants using the floral dip method (Clough and Bent, 1998) supplemented with vacuum infiltration. Since the construct had a *bar* selectable marker gene that confers resistance to the herbicide Basta, seeds from T_0 plants were screened for the transgene by applying Basta on soil-germinated young seedlings. To select for transgenic lines emitting the expected TPS5 volatile sesquiterpenes, headspace volatiles were collected from detached leaves of the Basta survivors with SPME (solid phase microextraction) and analyzed by GC-MS (see *Volatile Collection and Analysis* for details). Transformation was confirmed additionally by PCR analysis.

Plant and Insect Material A. thaliana plants overexpressing one of the maize sesquiterpene synthases TPS10, TPS8, or TPS5 (Schnee et al., 2006; Fontana et al. unpublished data), and wild-type (ecotype Col-0) plants were grown in 7 x 7 x 8 cm single pots on fertilized potting soil in a climate chamber at 21°C, 55% relative humidity, and 150 μmol s^{-1} m^{-2} photosynthetically active radiation (PAR) under short day conditions (8 h light day^{-1}) in order to retard flowering and maximize rosette size.

Maize plants var. Delprim were grown in a climate chamber at 23±2°C, 60% r.h., 16 h light day^{-1}, and 900 μmol s^{-1} m^{-2} PAR. Eggs of the Egyptian cotton armyworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), were obtained from Syngenta rearing facilities (Stein, Switzerland). After emergence, larvae were reared on a wheatgerm-based artificial diet (also supplied by Syngenta) at room temperature. The solitary endoparasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) was reared as described in Turlings et al. (2004). Female and male adult parasitoids were kept together in plastic cages (30

x 30 x 30 cm) (MegaView Science Education Services Co. Ltd., Taiwan), at a ratio of approximately 2 : 1 in incubators at 25±1°C with 16 h light day^{-1} and supplied with moist cotton and honey. Mated, 2- to 5-d-old females were used in the olfactometer experiments.

Olfactometer Experiments The behavioral response of *C. marginiventris* females to the three maize sesquiterpene blends and their combinations was tested by using transgenic *A. thaliana* plants as odor sources in six-arm olfactometer experiments. The six-arm olfactometer (described in Turlings et al., 2004) was set with incoming air at 1.2 l min^{-1} per vessel. A portion of the air was pulled out from each vessel at 0.6 l min^{-1} through a volatile collection trap (see *Volatile Collection and Analysis*). Each of the six vessels either contained a plant as the odor source or was left empty. Purified, humidified air carried the volatile odors from the vessels to the corresponding arm of the olfactometer. When combinations of two or three terpene blends were tested, two or three *A. thaliana* plants were used as odor sources. The same number of plants was used in all the odor-carrying arms in order to have a comparable plant biomass in all the vessels. Plants used were all at the rosette stage, 4 to 6 wk-old. The transgenic plants were tested to confirm volatile emission prior to each experiment, and different confirmed individuals were chosen randomly for each experimental replicate (see *Volatile Collection and Analysis*).

Groups of 6 *C. marginiventris* females were released into the center of the olfactometer and were given 30 min to fly into one of the arms. Wasps that did not enter an arm within this time were considered as having made no choice. After the choice of the wasps had been recorded, the wasps were removed from the olfactometer. Each group of parasitoids tested was either naïve (with neither oviposition experience nor exposure to any of the tested odors) or experienced (for details on the number of releases per experience and experimental replicates, see Table 1). Oviposition experiences were given by placing a single female parasitoid in a glass enclosure that contained about 20 2nd- or 3rd-instar *S. littoralis* larvae and connected on one side to a glass vessel that contained a given odor source, which was either a transgenic *Arabidopsis* plant or a maize seedling. The wasps were allowed to oviposit in presence of the odor at least three times before they were removed from the enclosure. To induce volatile production in the maize seedling used for these experiments, plants were enclosed in a glass vial with a vented lid and one 3rd-instar *S. littoralis* caterpillar was allowed to feed overnight.

Every experiment was replicated 3 to 8 times. At the end of each replicate, all parts of the olfactometer were washed with water, acetone, and hexane, and the glass parts were dried in an oven at 250°C. In each replicate, 1 to 4 groups of 6 wasps with the same experience were released (Table 1). In total, four experiments were performed. The layout of the olfactometer for each experiment is schematically represented in Fig. 2.

Volatile Collection and Analysis All transgenic *A. thaliana* plants were screened for volatile production before being used in the olfactometer experiments. A leaf was detached from ca. 3-wk-old plants, put in a 1.5 ml glass vial, and incubated for about 20 min at 40°C with an SPME fiber (100 μm polydimethylsiloxane coating, Supelco, USA). The collected volatiles were then analyzed by GC-MS (GC: Hewlett-Packard 6890; carrier gas: He, 1 ml min^{-1}, splitless injection (injection T: 220°C), column: DB-5MS (5% diphenyl/95% dimethylsiloxane, 30 m x 0.25 mm x 0.25 μm film, J & W Scientific, Folsom, CA, USA), temperature program: 80°C to 180°C at 10°C min^{-1}, 180°C to 220°C at 100°C min^{-1}. MS: Hewlett-Packard 5973, quadrupole mass selective detector; transfer line T: 270°C; ionization potential: 70 eV; scan range: m/z 40–350).

During the second olfactometer experiment, volatiles were collected from the vessels in order to assess whether the quantity of volatiles affected the wasp

Table 1 Summary of details of the olfactometer experiments, including types of oviposition experience used for *Cotesia marginiventris* prior to experiment, number of releases per experience, number of experimental replicates, and total number of wasps used[a]

	Experiences					Releases per experience	Replicates of the experiment	Total number of wasps
	Naïve	TPS8	TPS10	TPS8 + TPS10	HI[b] maize			
Exp. 1	x	x				4	3	144
Exp. 2	x	x	x	x	x	1	8	240
Exp. 3			x			3 - 4	4	84
Exp. 4			x			5 - 6	7	216

[a] Six wasps were used in each release. Naïve wasps had no prior oviposition experience

[b] HI: Herbivore-induced

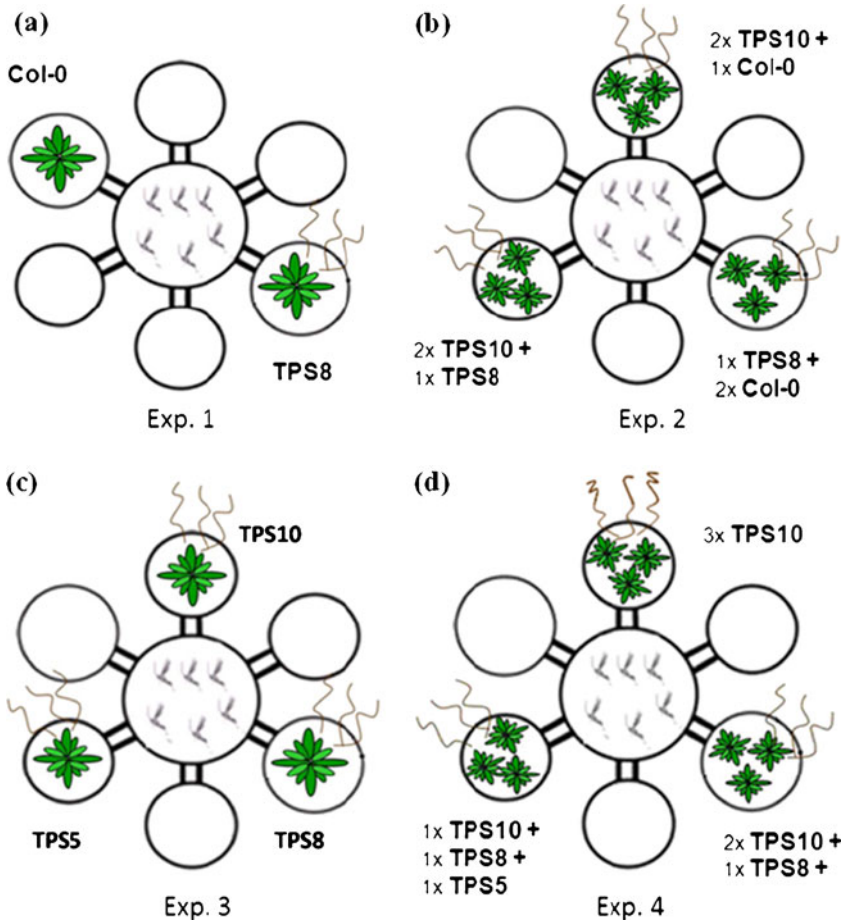


Fig. 2 Position of the odor sources in the olfactometer experiments (Experiment 1 **a**, Exp. 2 **b**, Exp. 3 **c**, Exp. 4**d**). The schematic plants represent *Arabidopsis thaliana* individuals, either transgenic or wild-type (ecotype Col-0)

responses. Odors were trapped with 25 mg Super-Q adsorbent (Alltech Associates, Inc., Deerfield, IL, USA), and eluted with 150 µl dichloromethane; 200 ng nonyl acetate were added as an internal standard. Volatiles were analyzed by GC-MS (GC: Agilent 6890 Series GC system G1530A, MS: Agilent 5973 Network Mass Selective Detector, electron impact mode, transfer line T: 230°C, ionization potential: 70 eV, scan range: m/z 33–280). The carrier gas was helium (1 ml min^{-1}). Samples were injected splitless at 230°C. We used an HP-1 column (Alltech Associates, polydimethylsiloxane, 30 m x 0.25 mm x 0.25 µm film), and the following temperature program: 40°C for 3 min, 40°C to 100°C at 8°C min^{-1}, 100°C to 200°C at 5°C min^{-1}, 5 min at 250°C. The relative quantity (%) of the sesquiterpenes was calculated based on their peak area relative to the peak area of the internal standard.

Statistical Analysis In order to analyze the influence of the odors tested and the experiences on the parasitoids' choices, a generalized linear model (GLM) with a log-link function and a quasipoisson error distribution was used (Turlings et al., 2004). "No choice" wasps were not included in the analyses. The model was fitted by maximum quasi-likelihood estimation, and its adequacy was assessed through likelihood ratio statistics and examination of residuals. Odor source and experience of the wasps were fitted to the model as fixed factors. We tested the interactions between the factors as well as the effect of the single factors on the wasps' choice. The effect of the odor was tested separately on naïve and experienced wasps in Exp. 1 and 2. The response of the wasps to the relative amounts of volatiles offered in Exp. 2 was assessed with the same GLM. All analyses were performed using the software package R (R Development Core Team (2008), R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0,URL http://www.R-project.org).

Results

The Constitutively Produced Maize Sesquiterpene Blend Is Attractive to Experienced C. marginiventris *Parasitoids (Exp.1)* Experienced *C. marginiventris* females that had had a previous oviposition experience in the presence of TPS8 volatiles were more attracted by *A. thaliana* plants producing

TPS8 terpenes than by wild-type plants ($F_{1,22}$=14.00, P=0.001), while naïve parasitoids did not show a preference for either plant ($F_{1,22}$=0.01, P=0.915) (Fig. 3a, b). Both naïve and experienced wasps preferred arms containing a plant to empty arms (naïve: $F_{2,69}$=14.40, P<0.001, experienced: $F_{2,69}$=45.63, P<0.001). Overall, 49% of the parasitoids responded to the plant odors (i.e., they entered an arm), while 45% made no choice (Fig. 3c).

Experienced C. marginiventris *Females Prefer the Full Sesquiterpene Blend of an Herbivore-Induced Maize Plant Including Constitutive Volatiles (Exp.2)* Since experienced *C. marginiventris* were attracted to the constitutively released TPS8 volatiles of a maize plant, we tested whether this attraction is similar to that of herbivore-induced TPS10 volatiles (Schnee et al., 2006) and whether there is any interaction between the two terpene blends. The volatile blend of herbivore-damaged maize seedlings contains about twice as many herbivore-induced TPS10 volatiles as constitutive TPS8 compounds. To mimic these proportions, two TPS10-emitting plants were combined with one TPS8-expressing plant (Fig. 2b, Exp.2). This blend was compared to the volatiles of two TPS10 plants combined with one Col-0 plant and to the volatiles of one TPS8 plant with two Col-0 plants to simulate the ratio of herbivore-induced to constitutive volatiles naturally emitted by maize plants. The combination of TPS10 and TPS8 sesquiterpenes was preferred by experienced wasps over the TPS8 volatiles alone (t=−2.65, P=0.009), but not over the TPS10 volatiles (t=−1.51, P=0.13) (Fig. 4b). Experienced *C. marginiventris* preferentially oriented towards arms carrying plant odors than towards empty arms ($F_{1,190}$=38.93, P<0.001). Naïve wasps did not significantly prefer any of the odors offered ($F_{2,21}$=0.85, P=0.44) (Fig. 4a), but they chose arms carrying plant volatiles more frequently than empty arms ($F_{2,21}$=0.85, P=0.03). The different oviposition experiences had no influence on the parasitoids' choice ($F_{3,188}$=0.24, P=0.87). The wasps' responsiveness was low: 40% of the parasitoids chose a plant odor, while 51% made no choice (Fig. 4c). The relative amount of the volatiles collected from the plant-containing vessels did not influence the distribution of the wasps in the olfactometer ($F_{1,22}$=6*10^{-4}, P=0.98).

Experienced C. marginiventris *Females Do Not Discriminate Between Different Maize Sesquiterpene Blends (Exp.3)* We tested the hypothesis suggested by the previous experiment that the association of volatiles with oviposition may not be dependent on specific sesquiterpenes. The parasitoids preferred arms containing a plant to empty arms ($F_{1,82}$=9.23, P=0.003), but none of the sesquiterpene blends offered was preferred over the others ($F_{2,39}$=0.58, P=0.56) (Fig. 5a). Again, the responsiveness was low: 30% of the parasitoids chose a plant odor, while 61% made no choice (Fig. 5b). These results indicated that *C. marginiventris* did not associate specific sesquiterpenes with oviposition and chose any of the three blends for host finding.

Experienced C. marginiventris *Females Tended to Orient towards More Complex Sesquiterpene Blends (Exp.4)* Because specific sesquiterpenes were not recognized by *C. marginiventris* (Exp.3), and because the blend of TPS8 and TPS10 sesquiterpenes combined were more attractive than each separately (Exp. 2), we tested whether greater complexity of the blend may increase its attraction to the wasps. Parasitoids preferred arms containing a plant to empty arms ($F_{1,95}$=6.72, P=0.011) (Fig. 6b). Although there was no statistically significant preference for any of the blends offered, the wasps tended to orient towards the most complex blend (TPS8 + TPS10 + TPS5) ($F_{2,45}$=2.67, P=0.08) (Fig. 6a). Overall, 25% of the parasitoids responded to the plant odors, while 66% made no choice.

Discussion

Herbivore-induced plant volatiles have been frequently demonstrated to play a role in the attraction of herbivore

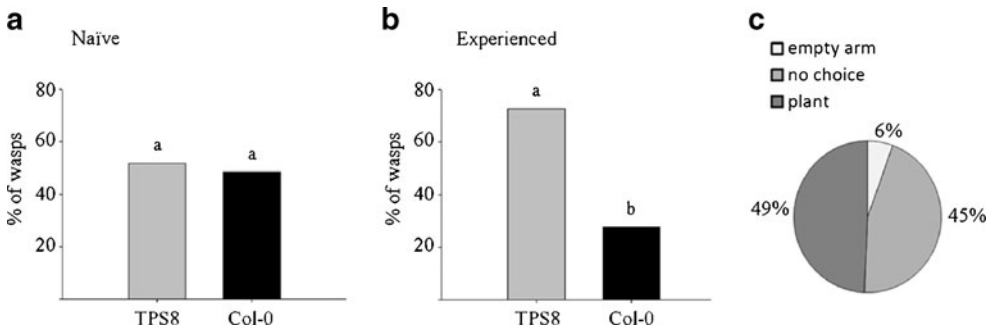


Fig. 3 Percentage of naïve **a** and experienced **b** *Cotesia marginiventris* females that were attracted to a terpene-emitting (TPS8) or a wild-type (Col-0) *Arabidopsis thaliana* plant in a six-arm olfactometer. Different letters represent a significant difference (P=0.001). Responsiveness of all the wasps tested is shown in **c**, where N=144=100%

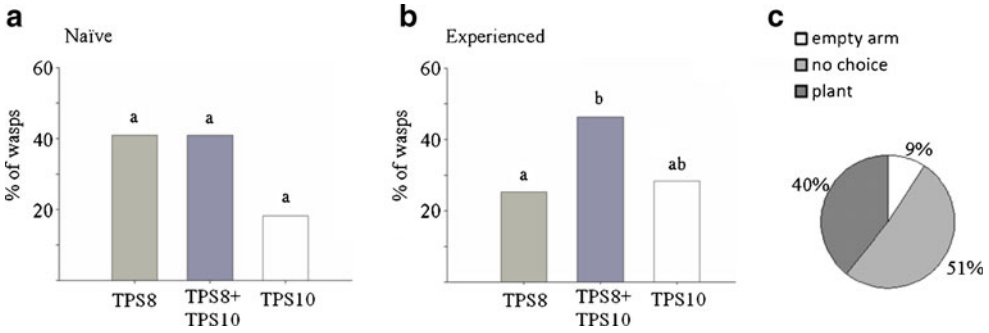


Fig. 4 Percentage of naïve **a** and experienced **b** *Cotesia marginiventris* females that were attracted to three maize sesquiterpene blends produced by transgenic *Arabidopsis thaliana* plants in six-arm olfactometer experiments. TPS8: sesquiterpene volatiles of an undamaged maize plant; TPS8 + TPS10: sesquiterpene volatiles of an herbivore-induced maize plant; TPS10: herbivore-induced sesquiterpenes of maize. Each transgenic line produces only a single blend; the mixture of TPS8 and TPS10 volatiles was obtained by combining TPS8- and TPS10-overexpressing plants. Different letters represent significant differences (P=0.009). Responsiveness of all the wasps tested is shown in **c**, where N=240=100%

enemies (Turlings et al., 1990; Takabayashi et al., 1991; Heil, 2008; Dicke, 2009). However, these studies usually do not take into account that enemies using herbivore-induced plant volatiles to search for their prey or hosts also come into contact with VOCs emitted by undamaged plant parts. A successful host finding strategy of an enemy might, therefore, involve both constitutive and herbivore-induced plant volatiles.

By utilizing *A. thaliana* plants genetically transformed with maize terpene synthases to emit blends of maize volatiles, we showed that females of *C. marginiventris*, a hymenopteran parasitoid that uses lepidopteran larvae as its hosts, can learn the constitutively released volatiles during an oviposition experience and be subsequently attracted to them. (Exp. 1, Fig. 3). Several other parasitoid species have also been shown to be attracted to odors from undamaged plants that support their hosts, including the aphid parasitoid *Aphidius funebris* Mackauer (Hymenoptera: Braconidae), which responded to uninfested *Centaurea nigra* L. (Asteraceae) (Pareja et al., 2007), and the lepidopteran parasitoids *Microplitis croceipes* and *Campoletis sonorensis*, which responded to cotton plant volatiles (Elzen et al., 1986, 1987). Here, we showed conclusively that specific maize sesquiterpene volatiles are involved in this attraction to undamaged plants.

In our experiments, experienced *C. marginiventris* females parasitoids were given a choice between terpene blends that reproduced: 1) the constitutive odor of an undamaged maize plant; 2) the odor produced by herbivore induction; or 3) the complete odor of an herbivore-infested plant, including both constitutive and induced volatiles. They showed a preference for the sesquiterpene blend that represented the combination of both constitutive and induced odors (Exp. 2, Fig. 4b). This shows that constitutively produced VOCs can act together with herbivore-induced VOCs to promote attraction of herbivore enemies. The constitutively emitted TPS8 terpenes may represent a background odor that indicates the general habitat of the host larvae. This background odor appears to reinforce the particular odor released by maize only upon herbivore feeding, thus enhancing parasitoid attraction. Such a positive

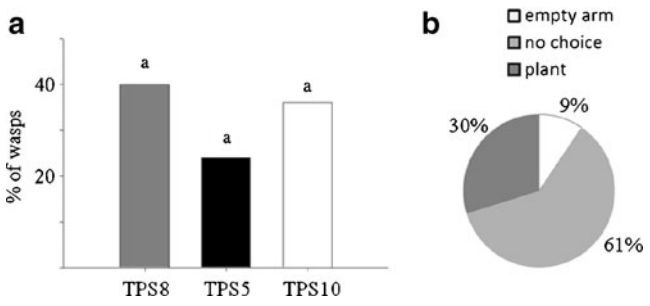


Fig. 5 Percentage of *Cotesia marginiventris* females that were attracted to sesquiterpene blends from maize produced by three lines of transgenic *Arabidopsis thaliana* plants in six-arm olfactometer experiments **a**. The terpene composition of the blends is detailed in Fig. 1. Responsiveness of all the wasps tested is shown in **b**, where N=240=100%

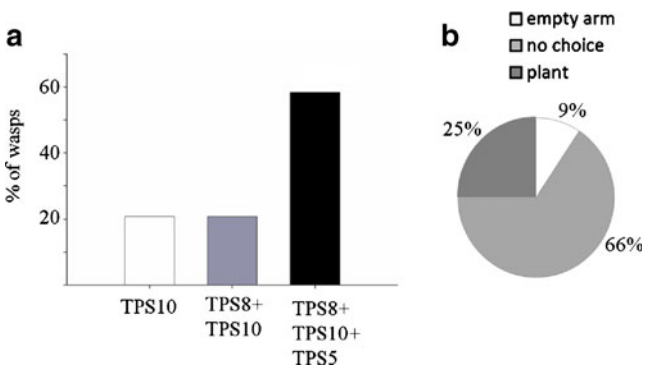


Fig. 6 Percentage of *Cotesia marginiventris* females that were attracted to sesquiterpene blends of increasing complexity in six-arm olfactometer experiments **a**. The odor sources were *Arabidopsis thaliana* plants each transformed with one maize sesquiterpene synthase gene (*tps8* or *tps10* or *tps5*). Plants from different transgenic lines were combined to give the combinations of odor blends. Responsiveness of all the wasps tested is shown in **b**, where N=96=100%

interaction of volatiles cues also has been described for *Leptopilina boulardi* (Barbotin, Carton & Kelner-Pillault) (Hymenoptera: Eucoilidae), a larval parasitoid of *Drosophila* species, which is attracted to the odors of pear and banana fruits (Couty et al., 1999). When this odor was provided together with damp filter papers impregnated with the smell of *Drosophila* flies, the parasitoids significantly preferred this combination to the smell of the flies alone. The importance of background odor for parasitoid host searching also has been highlighted by Mumm and Hilker (2005) in their study with the egg parasitoid *Closterocerus ruforum*, which did not respond to the signal of oviposition-induced pine twigs ((*E*)-β-farnesene) unless it was offered in combination with the odor of an uninfested twig. Another interesting interaction between odor blends has been reported by Fukushima and coworkers (2002), who found a synergistic effect in the attraction of conditioned *Cotesia kariyai* (Hymenoptera: Braconidae) parasitoids to a blend of four volatiles typically emitted after herbivory (geranyl acetate, (*E*)-β-caryophyllene, (*E*)-β-farnesene, and indole), and a non-specific blend made up of three GLVs, β-myrcene, and linalool. A limitation of this and other studies on volatiles lies in the authenticity of such partial plant volatile blends that are artificially constructed by mixing purified compounds, and may lack critical minor compounds. The use of transgenic *A. thaliana* plants transformed with TPS genes resulted in the formation of both major and minor components of the sesquiterpene blend, allowing a more accurate evaluation of its ecological function. Minor constitutents of volatile blends sometimes have been shown to play a disproportionate role in insect attraction (Leal, 2005, and references therein). In the present study, minor constituents other than sesquiterpenes could conceivably influence parasitoid attraction, but these did not differ among the *A. thaliana* lines tested (Suppl. Fig. 1).

The preference of experienced *C. marginiventris* females for the combination of two terpene blends observed in Experiment 2 also could be explained by the fact that more complex mixtures of terpenes are more attractive to these wasps. We tested, therefore, the effect of mixture complexity on *C. marginiventris* by giving females the choice between three terpene blends with an increasing number of components (Exp. 4). Here, the parasitoids tended to orient towards the most complex blend, suggesting that the number of compounds of a terpene mixture may play a role in attraction. Similar results have been obtained in field experiments where the number of insect herbivores attracted by a volatile blend was positively correlated with the number of chemicals present in the blend (Szendrei and Rodriguez-Saona, 2010). Complex volatile blends are known to play important roles in plant-insect interactions besides attraction of herbivore enemies. In a recent study, Riffell and coworkers investigated the perception and behavioral response of the moth *Manduca sexta* L. (Lepidoptera: Sphingidae) to the floral volatiles of the Sacred Datura (*Datura wrightii* (Regel), Solanaceae) (Riffell et al., 2009). Of the over 60 components of the floral scent, many of them terpenes, nine elicited a neural response in the moth, and, among these, four were monoterpenes (linalool, nerol, β-myrcene, and geraniol), and two were sesquiterpene hydrocarbons ((*E*)-β-caryophyllene and (*E,E*)-α-farnesene). Strikingly, these nine compounds were attractive to the moths only when offered as a mixture, but not when tested individually.

Theoretically, the preference of experienced *C. marginiventris* for the combination of two sesquiterpene blends observed in Experiment 2 also could be due to a higher total amount of volatiles, which leads to a stronger response. Previous studies indicate that the quantity of odors can be positively correlated to the attractiveness for different parasitoid species (Vaughn et al., 1996; Hoballah et al., 2002; Turlings et al., 2004; Kugimiya et al., 2010). However, in our work, the abundance of volatiles released in the olfactometer did not appear to influence the choice of the parasitoid (see *Results*, Exp. 2).

The parasitoid wasps used in our experiments did not prefer the TPS10 sesquiterpene mixture that they were trained on over the sesquiterpene mixtures formed by TPS8 and TPS5, although there are no major sesquiterpenes common to the three odors. This suggests that *C. marginiventris* may be attracted by the presence of sesquiterpenes in general, and does not distinguish among individual compounds. A study on *M. croceipes* investigated the ability of this egg parasitoid to distinguish among aliphatic alcohols differing in carbon chain-length and the position of functional groups (Meiners et al., 2002). The results suggested that a difference of at least two C atoms is necessary for wasps to make a choice between two aliphatic alcohols. It is possible that *C. marginiventris*, like *M. croceipes*, does not distinguish among mixtures of sesquiterpene hydrocarbons that all contain 15 carbon atoms, and, therefore, did not choose among the different blends offered, once they had learnt one of them.

The responsiveness of the parasitoids to the odors offered seemed to be low in our experiments, ranging from 25-49% (Figs. 3b, 4c, 5b, 6b). However, a responsiveness of *C. marginiventris* females of 42% was recorded previously by Turlings et al. (2004) in experiments with a six-arm olfactometer. An even lower responsiveness was found by Girling et al. (2006) in Y-tube olfactometer experiments with an aphid parasitoid (*Diaretiella rapae* (M'Intosh) (Hymenoptera: Aphiididae)) and *A. thaliana* plants. In this study, the percentages of parasitoids responding to the odors offered were as low as 22.5%. From this result, the authors concluded that the corresponding odors were not especially attractive to *D.*

rapae. On the other hand, Schnee et al. (2006) reported a responsiveness of 83% in olfactometer experiments with *C. marginiventris* and sesquiterpene-emitting TPS10 *A. thaliana* plants. Such a high responsiveness compared to the one we recorded could be due to the fact that the experiments were performed at different times of the year (Schnee et al. (2006) in spring, the experiments presented here in summer or in winter).

Studies on naïve *C. marginiventris* females that utilize the complete herbivore-induced volatiles of a maize plant demonstrated a responsiveness of up to 80% (D'Alessandro and Turlings, 2005). These high rates of attraction to the complete blend might be due to the fact that this contained one or more volatile compounds not present in our blends that may not have even been identified yet (D'Alessandro and Turlings, 2005; D'Alessandro et al., 2009). To fully understand the importance of volatile compounds in the host finding of *C. marginiventris*, bioassays should be carried out with additional constitutive host volatiles, such as monoterpenes and GLVs. Further tests with other parasitoids with different degrees of host specialization should give even more insight into the relative importance of constitutive vs. herbivore-induced compounds.

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Variation in Plant Defense against Invasive Herbivores: Evidence for a Hypersensitive Response in Eastern Hemlocks (*Tsuga canadensis*)

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Abstract Herbivores can trigger a wide array of morphological and chemical changes in their host plants. Feeding by some insects induces a defensive hypersensitive response, a defense mechanism consisting of elevated H_2O_2 levels and tissue death at the site of herbivore feeding. The invasive hemlock woolly adelgid *Adelges tsugae* ('HWA') and elongate hemlock scale *Fiorinia externa* ('EHS') feed on eastern hemlocks; although both are sessile sap feeders, HWA causes more damage than EHS. The rapid rate of tree death following HWA infestation has led to the suggestion that feeding induces a hypersensitive response in hemlock trees. We assessed the potential for an herbivore-induced hypersensitive response in eastern hemlocks by measuring H_2O_2 levels in foliage from HWA-infested, EHS-infested, and uninfested trees. Needles with settled HWA or EHS had higher H_2O_2 levels than control needles, suggesting a localized hypersensitive plant response. Needles with no direct contact to settled HWA also had high H_2O_2 levels, suggesting that HWA infestation may induce a systemic defense response in eastern hemlocks. There was no similar systemic defensive response in the EHS treatment. Our results showed that two herbivores in the same feeding guild had dramatically different outcomes on the health of their shared host.

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Introduction

The rapid expansion of global trade and transport links has been accompanied by a concomitant increase in the number of invasive species and the threat they pose to native ecosystems (Mooney and Cleland, 2001). As the number of biological invasions increases, the potential for interactions among invasive species will become more likely. Since invasive species tend to have high growth rates and population densities (Sakai et al., 2001), the likelihood and importance of these interactions is great (Denno et al., 1995). Such interactions are especially likely among specialist sessile herbivores that cannot emigrate in response to changes in host quality induced by competing insect species (Karban, 1989).

Herbivores can have differing effects on their host plants if one species, but not another, induces changes in plant physiology. Infestation by one herbivore may lead to the systemic induction of plant defenses and reduce the plant's susceptibility to subsequent invaders (Karban, 1990; Alborn et al., 1996; Bezemer et al., 2003). Some herbivores also are able to directly manipulate host plant metabolism and nitrogen allocation to their benefit, changes that can lead to the induction of systemic effects (Karban and Agrawal, 2002). Herbivores may be particularly harmful to their host if they are capable of inducing a pronounced hypersensitive response (HR). The HR is a plant defense response that increases reactive oxygen species, including hydrogen peroxide (H_2O_2), superoxide anions, and hydroxyl radical levels, and induces cell death in herbivore-damaged areas that isolates and starves invading organisms (Heath,

2000; Liu et al., 2010). Such localized tissue death usually is a response to sessile herbivores (Karban and Baldwin, 1997) and reduces the negative effects of insects such as balsam woolly adelgids, bark beetles, woodwasps, and gall midges (Fernandes, 1990; Ollerstam and Larsson, 2003). The hypersensitive response also defends plants against gall formation by isolating the galling insects (Fernandes and Negreiros, 2001).

The introduced hemlock woolly adelgid (*Adelges tsugae*; HWA) is causing mortality of eastern hemlocks along the east coast of North America (Orwig et al., 2002). A second invasive herbivore, the elongate hemlock scale (*Fiorinia externa*; EHS), also feeds on eastern hemlocks in North America (McClure, 1977). Although both species are sap-sucking insects that are considered pests, they have different impacts on their shared host (Preisser and Elkinton, 2008). In a multi-year experiment, Miller-Pierce et al. (2010) found that infestation with HWA, but not EHS, significantly decreased tree growth. While mature trees infested with HWA can die in as few as 4 years, mortality rarely occurs as a result of EHS infestation (McClure, 1980, 1991). The disproportionate hemlock mortality observed in response to HWA feeding has led to the hypothesis that HWA might have a systemic effect on the plant (Young et al., 1995), resulting in widespread tissue necrosis and tree mortality. We tested this hypothesis by measuring foliar H_2O_2, indicative of a hypersensitive response, in eastern hemlocks that were infested with HWA, EHS, or neither insect. Since plants often respond to sessile herbivores by mounting localized defenses (Fernandes, 1990), we expected both insects to induce a localized response at the site of infestation. We hypothesized that HWA settlement would induce a stronger HR in hemlock than EHS settlement, and that the HWA-induced HR would be detectable even in undamaged tissue (a 'systemic' response). Since a localized HR is marked by H_2O_2 exclusively surrounding the site of feeding, H_2O_2 accumulation in tissues not being directly fed upon would suggest the presence of a magnified HR in HWA-infested trees.

Methods and Materials

Our research took advantage of a pre-existing experimental manipulation intended to assess the long-term responses of hemlock trees to infestation with HWA or EHS (Miller-Pierce et al., 2010). While both projects sampled the same trees, our research question (assessment of an insect-induced hypersensitive response in hemlocks) differed substantially from those addressed in the initial research project (long-term changes in plant growth and C:N ratio in response to HWA and EHS infestation; Miller-Pierce et al., 2010).

Plant Material Hemlock saplings (1–1.5 m in height) were collected in April 2007 from the understory of a hemlock forest in Pelham, Massachusetts, USA. This area was outside the range of both HWA and EHS when the saplings were collected, and the saplings and surrounding trees were inspected prior to removal to ensure the absence of both insects. Saplings were transplanted into a field surrounded by a deer-proof fence at the University of Rhode Island's East Farm (Kingston, RI, USA). They were planted in a grid pattern and randomly assigned to each of 3 experimental treatments: HWA only, EHS only, or control (neither insect). Details of the insect inoculations are contained in Miller-Pierce et al. (2010); briefly, we used standard protocols (Butin et al., 2007) to inoculate saplings in the HWA and EHS treatments with the appropriate insect in May (for HWA) or June (for EHS) of every year from 2007 through 2010. From 2007 to 2008, trees in the HWA and EHS treatments had similar densities (2007–2008 mean: 0.97 and 0.93 insects/cm for HWA and EHS, respectively; Miller-Pierce et al., 2010), but in 2009 trees in the EHS treatment had higher densities (0.3 and 2.3 insects/cm for HWA and EHS, respectively; Miller-Pierce et al., 2010). To prevent contamination between treatments, each tree was caged during the time of year in which crawlers could be dispersed by wind.

In June 2010, we sampled foliage from 7 HWA-infested trees, 6 EHS-infested trees, and 8 uninfested control trees (21 total trees). We harvested four branches from each tree; each branch consisted of a single leader of new growth emerging from a ≥15 cm section of old growth. Each branch was bagged and returned to the lab where we recorded the number of HWA or EHS and the number of needles on the terminal 4 cm of new growth. All sampled branches in the insect treatments had settled HWA or EHS on the old growth. The spring generation of HWA had emerged, but had not yet settled on old growth. Because the new growth on each branch was produced after the first generation of HWA had emerged and begun to settle on old growth and prior to the emergence of EHS, there were no settled insects on any of the new growth; a careful examination of the new growth directly adjacent to the old growth on each branch confirmed that no insects had settled in this area.

Detection of H_2O_2 in Needles Detection of H_2O_2 involved a combination of chemical treatment and image analysis looking for a stain that is present if H_2O_2 is present in the needle. We removed the first two pairs of needles both above and below the base of new growth on each branch, giving 4 old-growth and 4 new-growth needles per branch. A DAB (1 mg ml^{-1} 3,3'-diaminobenzidine) solution was prepared following a protocol modified after Thordal-Christensen et al. (1997). The DAB was dissolved in DMSO (0.002% dimethyl sulfoxide) and then slowly added to water while stirring with a stir bar. Each needle was

placed in a separate 1.5 ml vial containing 1 ml of DAB-DMSO solution. We placed each vial in a bell jar and vacuum infiltrated each needle twice using Tween 20 as the surfactant (Katagiri et al., 2002). The vacuum was run to 26 in Hg and then left to run for 1 min. Following the second infiltration, the vials containing the needles were removed from the jar and allowed to sit in the DAB solution for 2 hr. To decolorize the needles and reveal the reddish-brown DAB polymer if H_2O_2 were present, we placed a cap with a small puncture on each vial and placed them in boiling ethanol (95%) for 10 min once a day for 3 d. Needles were stored in 95% ethanol.

Image Analysis We photographed treated needles using a PixeLink PL-A662 camera attached to an Olympus SZX12 microscope. Each digital image (1280×1020 pixels) was analyzed using Photoshop CS3 (Adobe; San Jose, CA, USA). In each needle, the number of stained pixels (indicative of H_2O_2 presence) was recorded and expressed as a percentage of the total needle area.

Statistical Analysis Response variables were needle area (in mm^2), the proportion of needle area that stained positive for the presence of H_2O_2, and needle density in the terminal 4 cm of new growth foliage. For both needle area and proportion of needle area stained, responses for the between-treatment analyses were calculated using the mean response of old- and new-growth foliage per tree for each treatment (21 replicates per foliage type, 42 total replicates). Because needle density was measured only on new growth foliage, there were 21 replicates in total for this variable. Data were square-root transformed when necessary to meet the assumptions of normality; variances were homogenous between treatments. We used ANOVA to test for the main effects of treatment, foliage age (old vs. new growth), and their interaction. Where appropriate, we performed means separation tests using Tukey's HSD.

Responses for the within-treatment analyses were calculated using the mean response of old- and new-growth foliage per branch per tree for each treatment (4 branches per tree, 28 replicates per foliage type in the HWA treatment, 24 replicates per foliage type in the EHS treatment). We used MANOVA to test for the main effects of insect density, foliage age, and tree, as well as an insect density*foliage age interaction. Statistical analyses were performed using JMP 6.0.2 (SAS Institute 2007).

Results

Between-Treatment Analyses Needles from HWA-infested trees had higher H_2O_2 levels than EHS and control treatments (Fig. 1a; $F_{2,36}=37.9$, $P<0.001$). In the HWA treatment, 25.2±2.30% (SE) of the needle area contained H_2O_2. Mean H_2O_2 levels in the EHS and control treatments were substantially lower (10.6±2.50% and 6.9±2.15% of total needle area, respectively).

Across all treatments, old-growth (infested) needles had higher H_20_2 levels than new-growth needles (18.5±2.44% and 9.4±1.91%, respectively; $F_{1,36}=30.9$, $P<0.001$). There was also a significant treatment*foliage age interaction ($F_{2,36}=3.34$, $P=0.047$). On old-growth foliage, both HWA and EHS showed an increase in H_2O_2 levels relative to the control (Tukey's HSD at $P=0.05$; Fig. 1a). On new-growth foliage, however, only trees in the HWA treatment showed increased H_2O_2 levels (Tukey's HSD at $P=0.05$; Fig. 1a).

Although there were no between-treatment differences in needle density ($F_{2,18}=0.33$, $P=0.72$), overall needle size differed between treatments (Fig. 1b; $F_{2,36}=4.03$, $P<0.026$). Needles in the control treatment were significantly larger than needles in the EHS treatment (13.52±0.61 mm^2 and 10.98±0.70 mm^2, respectively; Tukey's HSD at $P=0.05$). Needles in the HWA treatment averaged 11.81±0.65 mm^2 and did not differ from the control (Tukey's HSD at $P=0.05$).

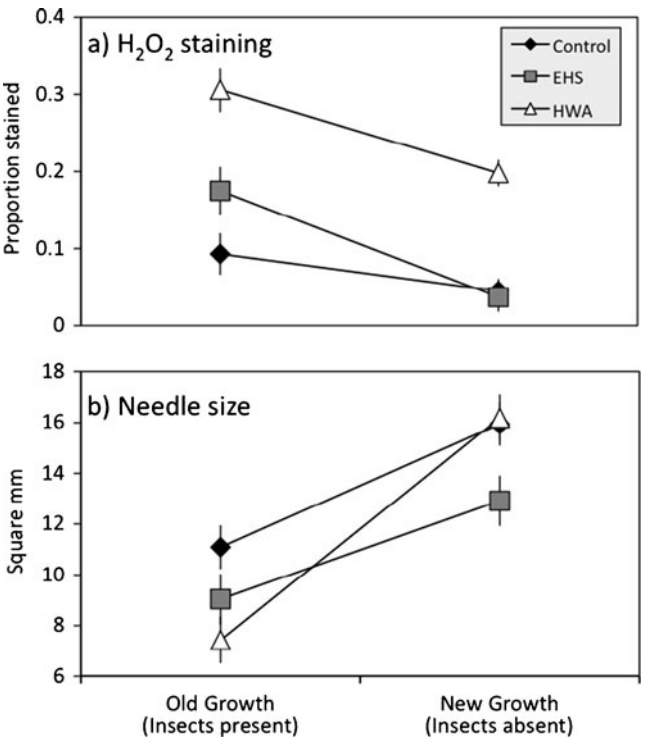


Fig. 1 Response of old-growth needles (herbivores present in hemlock woolly adelgid, 'HWA', and elongate hemlock scale, 'EHS', treatments) and new-growth needles (herbivores absent in all treatments) to infestation with HWA, EHS, or neither herbivore. Top panel (A): Proportion of needle area stained with DAB polymer, an indicator of H_2O_2. Bottom panel (B): Needle area (mm^2). Control treatment: Black diamonds, HWA present: White triangles, EHS present: Gray squares

Age had an effect on needle size ($F_{1,36}$=59.4, P<0.001). Across all treatments, old-growth needles were significantly smaller than new-growth needles (9.28±0.53 mm^2 and 15.16±0.53 mm^2, respectively). There was also a significant treatment*foliage age interaction ($F_{2,36}$=3.82, P=0.031; Fig. 1b). The proportional increase in needle size from old- to new-growth was similar in the control and EHS treatments, but not in the HWA treatment. Although old-growth needles in the HWA treatment were 33% smaller than old-growth control needles, new-growth needles in the HWA treatment were 1.5% larger than control new-growth needles (Fig. 1b).

Within-Treatment Analyses There was a positive correlation between HWA density and H_2O_2 levels ($F_{1,46}$=16.8, P<0.001; Fig. 2a). In contrast, there was no correlation between EHS density and H_2O_2 levels ($F_{1,39}$=0.56, P=0.46; Fig. 2b).

Although there was no correlation between HWA density and needle size ($F_{1,46}$=0.11, P=0.74), there was a positive correlation between EHS density and needle size ($F_{1,39}$=9.52, P=0.004). There was no relationship between insect density and needle density in either the HWA or EHS treatments (HWA: $F_{1,25}$=1.06, P=0.31; EHS: $F_{1,21}$=1.22, P=0.28).

Discussion

Our research showed that invasive herbivores with similar feeding modes can have very different effects on their shared host plant. Infestation by both hemlock woolly adelgid (HWA) and elongate hemlock scale (EHS) increased H_2O_2 levels relative to control hemlock needles, suggesting the presence of a localized hypersensitive response ('HR'; Fig. 1a). Although both insects induced a localized HR, needles with settled HWA at the base responded more strongly to insect settlement. After a year of HWA feeding, these old growth needles contained more H_2O_2 and were smaller than needles in the EHS or control treatments (Fig. 1b). The presence of HWA, but not EHS, also induced H_2O_2 accumulation in new growth needles that had no direct contact to feeding insects. Furthermore, HWA density on old growth needles was positively correlated with H_2O_2 accumulation in new growth needles (Fig. 2a), while EHS feeding was not (Fig. 2b). Since the HR is associated with cell death, the induction of a systemic hypersensitive response by HWA may partially explain why HWA causes more damage than EHS to eastern hemlocks.

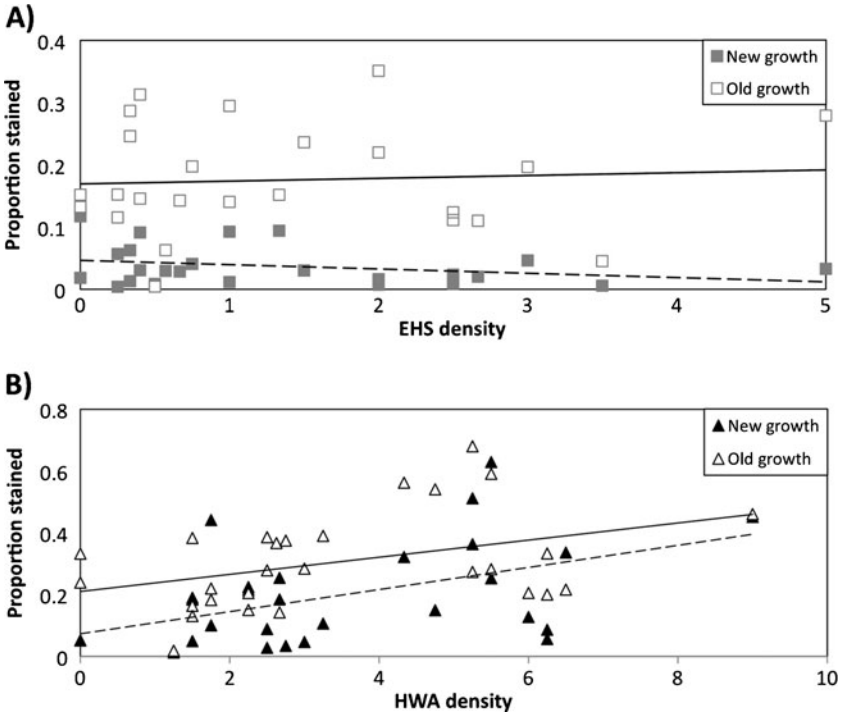


Fig. 2 Within-treatment correlation between settled insect density (either hemlock woolly adelgid, 'HWA', or elongate hemlock scale, 'EHS') on old-growth foliage and proportion of needle stained with DAB polymer. Top panel (A): Correlation between EHS densities on old-growth foliage and proportion of needle stained with DAB polymer. Old-growth needles: Open gray squares, New-growth needles: Closed gray squares. Old-growth correlation: Solid line ($F_{1,22}$=0.097, P=0.76), New-growth correlation: Dotted line ($F_{1,22}$=1.8, P=0.19). Bottom panel (B): Correlation between HWA densities on old-growth foliage and proportion stained with DAB polymer. Old-growth needles: Open black triangles, New-growth needles: Closed black triangles. Old-growth correlation: Solid line ($F_{1,26}$=4.83, P=0.37), New-growth correlation: Dotted line ($F_{1,26}$=7.91, P=0.009)

There are several possible explanations for the systemic effect of HWA settlement. Since measurements were made in old and new growth on the same branch, one possibility is that the H_2O_2 was produced in high quantities locally and spilled over into new growth foliage. The overproduction of defensive compounds could have been elicited by the mechanical damage caused by HWA stylet bundle insertion. This may be due to the slightly different feeding modes of the two insects. HWA settles on the petiole of the needle and feeds upon the xylem ray parenchyma cells, which function in nutrient storage (Young et al., 1995). In contrast, EHS settles on the underside of the needle and feeds upon mesophyll cells (McClure, 1980). Another possibility is that the defense compounds were elicited by some other factor related to HWA feeding (Young et al., 1995), as has been seen in several aphid species (Miles, 1999). Certain constituents in HWA saliva may be toxic at high levels of infestation, causing the hemlocks to initiate a defensive HR and produce large quantities of H_2O_2 that are carried into new growth tissues. Continued feeding by subsequent generations of HWA is common, as HWA has two generations while EHS has only one, and persistently high levels of H_2O_2 may lead to the suppression of bud formation after several years of HWA feeding (McClure, 1991).

The HWA-induced H_2O_2 also could have been produced in new growth needles *de novo* as a result of a magnified HR that extended even to tissues without settled herbivores. In order to be effective, a systemic response should provide systemic induced resistance to further herbivore attack. For example, Moore et al. (2003) found that increases in cell-wall peroxidase in *Rumex obtusifolius* reduce the amount of future herbivore damage by strengthening cell walls. A systemic hypersensitive response in response to pathogen infection and wounding has been found in several other plants (Alvarez et al., 1998; Orozco-Cardenas and Ryan, 1999), and Alvarez et al. (1998) suggested that the H_2O_2 was generated systemically and may function in induced immunity. Hemlocks mounting a successful systemic response could provide new growth needles with more protection from sessile herbivores. The widespread mortality of eastern hemlocks suggests, however, that this response does not provide adequate protection from HWA. Bonello et al. (2006) proposed that if an invader can persist despite local defenses, the plant will become increasingly stressed by the cost of defense and will become susceptible to future attack. Since high HWA densities induce a magnified HR in infested tissues, a defensive response that would otherwise be beneficial could become costly and lethal to the host plant (Bi and Felton, 1995).

Data on needle size also demonstrate that these herbivores also have different effects on plant morphology. Although old growth HWA needles were the smallest, new-growth needles on HWA-infested branches were as large as controls (Fig. 1b). EHS and control needle size increased at the same rate from old to new growth, while HWA needle size increased at a much greater rate. The increase in needle size could be a result of mobilization of plant nutrients away from stressed older needles and into new growth. A review by Trumble et al. (1993) outlines similar results in other plants, in which leaves remaining after herbivore defoliation become larger in length and weight. This hypothesis is supported by research exploring the amino acid profile of HWA- or EHS-infested vs. uninfested hemlocks that found higher amino acid levels in new-growth needles on HWA-infested trees relative to new-growth needles on EHS-infested trees (Gomez et al. unpublished data). Remobilization of nutrients away from herbivore feeding and the site of damage may be a strategy to tolerate insect infestation (Gómez et al., 2010). Because the summer generation of HWA feeds almost exclusively on new growth tissue, HWA may benefit from the production of these nutrient-rich new-growth needles. While additional research is necessary to determine the precise mechanism(s) underlying our findings, at a broader level the work illustrates that species-specific differences in herbivore feeding can substantially affect plant responses to biological invasions.

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Antifeedant and Termiticidal Activities of 6-Alkoxycoumarins and Related Analogs Against *Coptotermes formosanus* Shiraki

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Abstract We synthesized 23 6-alkoxycoumarin derivatives, 20 of which are novel compounds. The structures of all compounds were confirmed by NMR, MS, and elemental analysis, and their antifeedant and termiticidal activities against *Coptotermes formosanus* Shiraki were examined. In a no-choice test, 6-(2-pentynyloxy)coumarin (**2v**), 6-(2-butynyloxy)coumarin (**2u**), 6-(2-octynyloxy) coumarin (**2w**), and 6-methoxycoumarin (**2a**), demonstrated high termiticidal activity at a concentration of 10 μmol. At a concentration of 5 μmol, 6-(2-butynyloxy)coumarin (**2u**) produced the highest mortality among the compounds tested. On the other hand, all of the 6-alkoxycoumarins showed antifeedant activity at both concentrations, except 6-octadecyloxycoumarin (**2j**) that was inactive at 5 μmol. Among the 23 compounds and the control, 6-ethoxycoumarin (**2b**), 6-isopropoxycoumarin (**2d**), and 6-isobutoxycoumarin (**2f**) exhibited the highest antifeedant activity with no mass loss (0.00%) at a concentration of 10 μmol. Our findings indicate that the presence of alkenyloxy and alkynyloxy groups was important for the termiticidal activity, while the incorporation of alkoxy groups with longer alkyl chains tended to reduce both the termiticidal and antifeedant activities. Furthermore, short chain alkoxy and arylalkoxy-substituted analogs showed good antifeedant activity, but methoxy groups on the benzene ring had a negative effect.

Key Words 6-alkoxycoumarins · *Coptotermes formosanus* Shiraki · 6-(2-pentynyloxy)coumarin · 6-isobutoxycoumarin · Antifeedant · Termiticidal

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Introduction

Coptotermes formosanus Shiraki is a subterranean termite responsible for major destruction of wooden materials. The diet of *C. formosanus* consists of anything that contains wood fiber, including residential and commercial structures and live plants (Meyer, 2005). Chemical treatments are the most important and widely used methods to reduce the infestation of termites. Several termiticides containing active ingredients, including bifenthrin, chlorfenapyr, cypermethrin, fipronil, imidacloprid, and permethrin are registered for termite control around the world under various brand names (Verma et al., 2009).

Environmental and health problems related to the use of synthetic insecticides have increased the need for less toxic and more environmentally friendly insect control substances. New methods for termite control are being developed continually by researchers. Plant-derived natural products, entomopathogenic fungi (Krutmuang and Mekchay, 2005; Mo et al., 2006), bacteria (Devi et al., 2007), nematodes (Weeks and Baker, 2004), physical barriers, and bait technology (Culliney and Grace, 2000; Evans and Gleeson, 2006; Verma et al., 2009) are some of the alternative methods. The use of botanicals as termiticides, termite

antifeedants, or termite repellents has been reported, and these include several quinones (plumbagin, isodiospyrin, and microphyllone) from *Diospyros sylvatica* (Ganapaty et al., 2004), flavonoids (castillen D and castillen E) from *Lonchocarpus castilloi* Standley (Reyes-Chilpa et al., 1995), limonoids (deacetylgedunin, gedunin, salanin, nimbandiol, azadiradione, and deacetylsalannin) from *Azadirachta indica* A. Juss. (Serit et al., 1992), and sesquiterpenes (sugikurojinols A and B) from the black heartwood of *Cryptomeria japonica* D. Don. (Arihara et al., 2004).

The relationship between chemical structure and antitermite activity for some of these active compounds has been investigated. For example, a study of latifolin and its derivatives suggested that the phenolic hydroxyl group at the C-5 position of the A ring is responsible for the termiticidal and antifeedant activities (Sekine et al., 2009). The termite antifeedant activity of some flavonoids (quercetin, taxifolin, and naringenin) also was influenced by the position of hydroxyl groups (Ohmura et al., 2000). Furthermore, Zhu et al. (2003) evaluated the repellency of eight valencenoid derivatives against *C. formosanus*, and they found that 1,10-dihydronootkatone was the most potent. The results showed that the ketone group at the 2-position of these valencenoid analogs was important for termite repellency. Recently, we confirmed the structure–activity relationship (SAR) of coumarin derivatives with antitermite activity (Adfa et al., 2010b). Coumarin and its derivatives were found to exhibit a broad spectrum of biological and pharmacological activities, such as molluscicidal (Schönberg and Latif, 1954), nematicidal (Takaishi et al., 2008), rodenticidal (Ishizuka et al., 2008), insecticidal, and antihelmintic properties (Soine, 1964). They also have exhibited anticoagulant, antibacterial, antioxidant, anti-inflammatory, and anti-cancer effects (Murray et al., 1982).

Our previous paper investigated the SAR of methoxy and hydroxy groups at C-6 and C-7 positions of the coumarin skeleton on termiticidal and antifeedant activities against *C. formosanus*. Scopoletin, isolated from *Protium javanicum* Burm. f. contains an OCH_3 and an OH group at C-6 and C-7 positions, respectively. Scopoletin exhibited the strongest termiticidal and antifeedant activities among the 10 compounds tested, followed by 6-methoxycoumarin, 6-hydroxycoumarin, and 7-hydroxycoumarin. The other compounds showed only weak or moderate activities. These results suggested that the methoxy or hydroxy groups at the C-6 and/or C-7 positions of the coumarin skeleton are important for termiticidal and antifeedant activities (Adfa et al., 2010b). To identify molecules with greater activity, we continued this study, investigating the effect of various substitutions on the oxygen at the C-6 position of the coumarin skeleton on antitermite activity. In this paper, we report the synthesis of 6-alkoxycoumarin analogs (**2a**–**2w**) and their antitermite properties against *C. formosanus*.

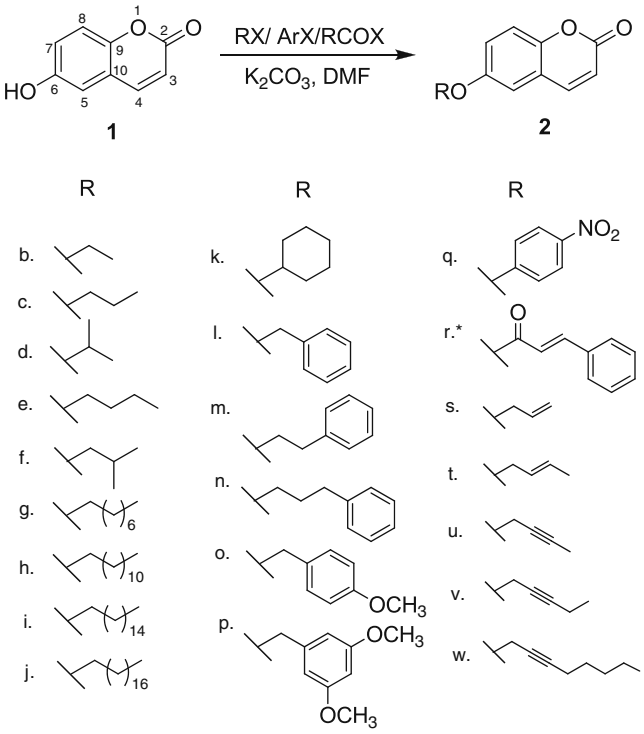
Methods and Materials

General Experimental Procedures ^{1}H and ^{13}C-NMR spectra were recorded on a JOEL ECA 600/400 spectrometer with tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded using a JOEL JMS-700/GI mass spectrometer. Column chromatography was performed on a neutral silica gel (Silica Gel 60N, spherical, neutral, 40–50 μm) (KANTO Chemical Co., Inc.). Melting points were determined on a Yanagimoto micro melting-point apparatus. Elemental analyses were determined on a YANAKO MTA-620. All chemicals were purchased from Sigma-Aldrich and Tokyo Chemical Industry Co., Ltd.

Synthesis Procedure and Spectral Data of 6-Alkoxycoumarins and Related Analogs A mixture of 6-hydroxycoumarin (**1**) (30 mg, 0.19 mmol), alkyl, aryl, or acyl halide (0.38 mmol), and potassium carbonate (79 mg, 0.57 mmol) in DMF (5.0 ml) was stirred at 55–85°C for times ranging from 0.5 h to overnight (Table 1). The reaction mixture was extracted with ethyl acetate, and the combined extracts were washed with water. The organic layer was dried over sodium sulfate and evaporated to dryness. The residue then was purified by column chromatography on silica gel with *n*-hexane/ethyl acetate (7:3) to yield the products (**2b**–**2q**, **2s**–**2w**). Instead of K_2CO_3, Et_3N (79 μl, 0.57 mmol) and DMAP (23.2 mg, 0.19 mmol) were used for the preparation of **2r** at 80°C for 1 h (Scheme 1 and Table 1). 6-Methoxycoumarin (**2a**) was synthesized according to the previous report (Adfa et al., 2010b).

6-Ethoxycoumarin (**2b**), colorless crystals, mp. 108°C (97% yield): ^{1}H-NMR (600 MHz, $CDCl_3$): δ 1.44 (3H, t, *J*= 6.9 Hz, H-2'), 4.06 (2H, q, *J*=6.9 Hz, H-1'), 6.42 (1H, d, *J*= 9.6 Hz, H-3), 6.91 (1H, d, *J*=2.7 Hz, H-5), 7.10 (1H, dd, *J*= 8.9 and 2.7 Hz, H-7), 7.26 (1H, d, *J*=8.9 Hz, H-8), 7.65 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 14.9 (C-2'), 64.3 (C-1'), 110.8 (C-5), 117.1 (C-3), 118.0 (C-8), 119.3 (C-10), 120.0 (C-7), 143.4 (C-4), 148.5 (C-9), 155.5 (C-6), 161.2 (C-2); FAB-MS m/z: 191 $[M+H]^+$ (matrix: mixture of dithiothreitol and dithioerythritol 3:1); Anal. Calcd for $C_{11}H_{10}O_3$: C, 69.49; H, 5.30. Found: C, 69.20; H, 5.56.

6-Propoxycoumarin (**2c**), colorless crystals, mp. 75°C (97% yield): ^{1}H-NMR (600 MHz, $CDCl_3$): δ 1.06 (3H, t, *J*=7.6 Hz, H-3'), 1.81–1.86 (2H, m, H-2'), 3.94 (2H, t, *J*= 6.9 Hz, H-1'), 6.42 (1H, d, *J*=9.6 Hz, H-3), 6.91 (1H, d, *J*= 2.8 Hz, H-5), 7.11 (1H, dd, *J*=8.9 and 2.8 Hz, H-7), 7.26 (1H, d, *J*=8.9 Hz, H-8), 7.65 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 9.6 (C-3'), 21.6 (C-2'), 69.4 (C-1'), 109.9 (C-5), 116.1 (C-3), 116.9 (C-8), 118.3 (C-10), 119.1 (C-7), 142.4 (C-4), 147.4 (C-9), 154.8 (C-6), 160.2 (C-2); FAB-MS m/z: 205 $[M+H]^+$ (matrix: mixture of dithiothreitol and dithioerythritol 3:1); Anal. Calcd for $C_{12}H_{12}O_3$: C, 70.57; H, 5.92. Found: C, 70.85; H, 6.25.



Scheme 1 Synthesis of 6-alkoxycoumarins and related analogs (**2b**–**2w**)

6-Isopropoxycoumarin (**2d**), colorless crystals, mp. 57°C (88% yield): ^{1}H-NMR (600 MHz, $CDCl_3$): δ 1.36 (6H (2 CH_3), d, *J*=6.2 Hz, H-2' and H-3'), 4.52–4.58 (1H, m, H-1'), 6.42 (1H, d, *J*=9.6 Hz, H-3), 6.93 (1H, d, *J*=2.8 Hz, H-5), 7.09 (1H, dd, *J*=8.9 and 2.8 Hz, H-7), 7.25 (1H, d, *J*=8.9 Hz, H-8), 7.65 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 20.0 (C-2' and C-3'), 71.1 (C-1'), 112.8 (C-5), 117.1 (C-3), 118.0 (C-8), 119.4 (C-10), 121.3 (C-7), 143.4 (C-4), 148.4 (C-9), 154.4 (C-6), 161.2 (C-2); FAB-MS m/z: 205 $[M+H]^+$(matrix: mixture of dithiothreitol and dithioerythritol 3:1); Anal. Calcd for $C_{12}H_{12}O_3$: C, 70.57; H, 5.92. Found: C, 70.42; H, 6.20.

6-Butoxycoumarin (**2e**), colorless crystals, mp. 99°C (99% yield): ^{1}H-NMR (600 MHz, $CDCl_3$): δ 0.99 (3H, t, *J*=7.6 Hz, H-4'), 1.48–1.54 (2H, m, H-3'), 1.79 (2H, quint., *J*=7.6 Hz, H-2'), 3.98 (2H, t, *J*=6.8 Hz, H-1'), 6.42 (1H, d, *J*=9.6 Hz, H-3), 6.91 (1H, d, *J*=2.8 Hz, H-5), 7.10 (1H, dd, *J*=8.9 and 2.8 Hz, H-7), 7.25 (1H, d, *J*=8.9 Hz, H-8),7.65 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 13.9 (C-4'), 19.3 (C-3'), 31.3 (C-2'), 68.6 (C-1'), 110.8 (C-5), 117.1 (C-3), 118.0 (C-8), 119.3 (C-10), 120.1 (C-7), 143.4 (C-4), 148.4 (C-9), 155.8 (C-6), 161.2 (C-2); FAB-MS m/z: 219 $[M+H]^+$ (matrix: mixture of dithiothreitol and dithioerythritol 3:1); Anal. Calcd for $C_{13}H_{14}O_3$: C, 71.54; H, 6.47. Found: C, 71.67; H, 6.76.

6-Isobutoxycoumarin (**2f**), colorless crystals, mp. 46–47°C (88% yield): ^{1}H-NMR (600 MHz, $CDCl_3$): δ 1.04 (6H (2CH_3), d, *J*=6.8 Hz, H-3' and H-4'), 2.07–2.14 (1H, m, H-2'), 3.74 (2H, d, *J*=6.2 Hz, H-1'), 6.42 (1H, d, *J*=9.6 Hz, H-3), 6.91 (1H, d, *J*=2.8 Hz, H-5), 7.11 (1H, dd, *J*=8.9 and 2.8 Hz, H-7), 7.25 (1H, d, *J*=8.9 Hz, H-8), 7.65 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 19.3 (C-3' and C-4'), 28.4 (C-2'), 75.2 (C-1'), 110.8 (C-5), 117.1 (C-8), 117.9 (C-3), 119.2 (C-10), 120.1 (C-7), 143.4 (C-4), 148.4 (C-9), 155.8 (C-6), 161.2 (C-2); Anal. Calcd for $C_{13}H_{14}O_3$: C, 71.54; H, 6.47. Found: C, 71.31; H, 6.63.

6-Octyloxycoumarin (**2g**), colorless crystals, mp. 60°C (98% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.89 (3H, t, *J*=6.9 Hz, H-8'), 1.19–1.41 (8H, br, H-4'–H-7'), 1.41–1.52 (2H, m, H-3'), 1.72–1.86 (2H, m, H-2'), 3.97 (2H, t, *J*=6.9 Hz, H-1'), 6.42 (1H, d, *J*=9.6 Hz, H-3), 6.91 (1H, d, *J*=2.8 Hz, H-5), 7.10 (1H, dd, *J*=8.9 and 2.8 Hz, H-7), 7.25 (1H, d, *J*=8.9 Hz, H-8), 7.65 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 14.2 (C-8'), 22.7 (C-7'), 26.1 (C-3'), 29.26 (C-5'), 29.32 (C-4'), 29.42 (C-2'), 31.9 (C-6'), 68.8 (C-1'), 110.8 (C-5), 117.0 (C-3), 117.9 (C-8), 119.2 (C-10), 120.0 (C-7), 143.4 (C-4), 148.4 (C-9), 155.7 (C-6), 161.2 (C-2); FAB-MS m/z: 275 $[M+H]^+$(matrix: m-nitrobenzyl alcohol); HRFABMS m/z: 275.1629 $[M+H]^+$ (calcd for $C_{17}H_{23}O_3$, 275.1647, matrix: PEG 400); Anal.

Table 1 Reaction conditions and yields of 6-alkoxycoumarins and related analogs (**2b**–**2w**)

Entry	R	Reaction conditions	Yield (%) **2**
1.	Ethyl	55°C, overnight	97 (**2b**)
2.	*n*-Propyl	80°C, 8 hr	97 (**2c**)
3.	Isopropyl	80°C, overnight	88 (**2d**)
4.	*n*-Butyl	55°C, overnight	99 (**2e**)
5.	Isobutyl	55°C, overnight	88 (**2f**)
6.	Octyl	80°C, 1 hr	98 (**2g**)
7.	Dodecyl	80°C, 1 hr	98 (**2h**)
8.	Hexadecyl	80°C, 1 hr	97 (**2i**)
9.	Octadecyl	80°C, 1 hr	96 (**2j**)
10.	Cyclohexyl	85°C, overnight	41 (**2k**)
11.	Benzyl	60°C, 1.5 hr	86 (**2l**)
12.	Phenetyl	80°C, overnight	82 (**2m**)
13.	3-Phenylpropyl	80°C, 2 hr	98 (**2n**)
14.	4-Methoxybenzyl	80°C, overnight	91 (**2o**)
15.	3,5-Dimethoxybenzyl	80°C, 5.5 hr	78 (**2p**)
16.	4-Nitrophenyl	55°C, overnight	62 (**2q**)
17.	Cinnamoyl	80°C, 1 hr	93 (**2r**)[a]
18.	Allyl	60°C, 10 hr	93 (**2s**)
19.	(*E*)-2-Butenyl	70°C, 3 hr	88 (**2t**)
20.	2-Butynyl	rt, 7 hr	92 (**2u**)
21.	2-Pentynyl	80°C, 1 hr	85 (**2v**)
22.	2-Octynyl	80°C, 0.5 hr	89 (**2w**)

[a] Et_3N and DMAP instead of K_2CO_3 were used

Calcd for $C_{17}H_{22}O_3$: C, 74.42; H, 8.08. Found: C, 74.29; H, 8.03.

6-Dodecyloxycoumarin (**2h**), white powder, mp. 69°C (98% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.88 (3H, t, *J*=6.9 Hz, H-12'), 1.18–1.39 (16H, br, H-4'–H-11'), 1.42–1.51 (2H, m, H-3'), 1.75–1.84 (2H, m, H-2'), 3.97 (2H, t, *J*=6.8 Hz, H-1'), 6.41 (1H, d, *J*=9.6 Hz, H-3), 6.91 (1H, d, *J*=2.8 Hz, H-5), 7.10 (1H, dd, *J*=9.2 and 2.8 Hz, H-7), 7.24 (1H, d, *J*=9.2 Hz, H-8), 7.65 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 14.2 (C-12'), 22.8 (C-11'), 26.1 (C-3'), 29.3, 29.4, 29.68, 29.73 (C-2'; C-4'–C-9'), 32.0 (C-10'), 68.8 (C-1'), 110.8 (C-5), 117.0 (C-3), 117.9 (C-8), 119.2 (C-10), 120.0 (C-7), 143.4 (C-4), 148.4 (C-9), 155.7 (C-6), 161.1 (C-2); HRFABMS m/z: 331.2296 $[M+H]^+$(calcd for $C_{21}H_{31}O_3$, 331.2273, matrix: PEG 400); Anal. Calcd for $C_{21}H_{30}O_3$: C, 76.33; H, 9.15. Found: C, 76.46; H, 9.45.

6-Hexadecyloxycoumarin (**2i**), white powder, mp. 72°C (97% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.88 (3H, t, *J*=6.4 Hz, H-16'), 1.21–1.51 (26H, br, H-3'–H-15'), 1.70–1.82 (2H, m, H-2'), 3.97 (2H, t, *J*=6.4 Hz, H-1'), 6.41 (1H, d, *J*=9.6 Hz, H-3), 6.91 (1H, d, *J*=2.8 Hz, H-5), 7.10 (1H, dd, *J*=8.9 and 2.8 Hz, H-7), 7.24 (1H, d, *J*=8.9 Hz, H-8), 7.65 (1H, d, *J*=9.6 Hz, H-4).; ^{13}C-NMR (100 MHz, $CDCl_3$): δ 14.2 (C-16'), 22.8 (C-15'), 26.1 (C-3'), 29.3, 29.5, 29.7, 29.76, 29.80 (C-2'; C-4'–C-13'), 32.0 (C-14'), 68.8 (C-1'), 110.8 (C-5), 117.0 (C-8), 117.9 (C-3), 119.2 (C-10), 120.0 (C-7), 143.4 (C-4), 148.4 (C-9), 155.7 (C-6), 161.1 (C-2); FAB-MS m/z: 387 $[M+H]^+$(matrix: m-nitrobenzyl alcohol); Anal. Calcd for $C_{25}H_{38}O_3$: C, 77.68; H, 9.91. Found: C, 77.62; H, 10.30.

6-Octadecyloxycoumarin (**2j**), white powder, mp. 76°C (96% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.88 (3H, t, *J*=6.4 Hz, H-18'), 1.18–1.51 (30H, br, H-3'–H-17'), 1.80 (2H, quint., *J*=6.9 Hz, H-2'), 3.97 (2H, t, *J*=6.4 Hz, H-1'), 6.42 (1H, d, *J*=9.6 Hz, H-3), 6.90 (1H, d, *J*=2.8 Hz, H-5), 7.10 (1H, dd, *J*=9.2 and 2.8 Hz, H-7), 7.25 (1H, d, *J*=9.2 Hz, H-8), 7.65 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 14.3 (C-18'), 22.8 (C-17'), 26.1 (C-3'), 29.3, 29.5, 29.7, 29.8 (C-2'; C-4'–C-15'), 32.1 (C-16'), 68.9 (C-1'), 110.8 (C-5), 117.1 (C-3), 117.9 (C-8), 119.3 (C-10), 120.1 (C-7), 143.4 (C-4), 148.4 (C-9), 155.7 (C-6), 161.2 (C-2); FAB-MS m/z: 415 $[M+H]^+$(matrix: m-nitrobenzyl alcohol); Anal. Calcd for $C_{27}H_{42}O_3$: C, 78.21; H, 10.21. Found: C, 78.40; H, 10.51.

6-Cyclohexyloxycoumarin (**2k**), colorless crystals, mp. 98–99°C (41% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.26-2.0 (10H, m, ($5CH_2$)-cyclohexyl), 4.21–4.27 (1H, m, H-1'), 6.41 (1H, d, *J*=9.6 Hz, H-3), 6.93 (1H, d, *J*=3.2 Hz, H-5), 7.10 (1H, dd, *J*=9.2 and 2.8 Hz, H-7), 7.25 (1H, d, *J*=9.2 Hz, H-8), 7.64 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 23.8 (C-3' and C-5'), 25.7 (C-4'), 31.8 (C-2' and C-6'), 76.6 (C-1'), 113.1 (C-5), 117.1 (C-3), 118.0 (C-8), 119.4 (C-10), 121.4 (C-7), 143.4 (C-4), 148.5 (C-9), 154.3 (C-6), 161.2 (C-2); Anal. Calcd for $C_{15}H_{16}O_3$: C, 73.75; H, 6.60. Found: C, 73.50; H, 6.42.

6-Benzyloxycoumarin (**2l**), colorless crystals, mp. 112°C (86% yield): ^{1}H-NMR (600 MHz, $CDCl_3$): δ 5.10 (2H, s, H-1'), 6.42 (1H, d, *J*=9.6 Hz, H-3), 6.99 (1H, d, *J*=2.8 Hz, H-5), 7.18 (1H, dd, *J*=8.9 and 2.8 Hz, H-7), 7.26 (1H, d, *J*=8.9 Hz, H-8), 7.34–7.44 (5H, m, Ar-H), 7.63 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 70.8 (C-1'), 111.5 (C-5), 117.2 (C-8), 118.1 (C-3), 119.3 (C-10), 120.3 (C-7), 127.6 (C-3 Ar and C-5 Ar), 128.4 (C-4 Ar), 128.8 (C-2 Ar and C-6 Ar), 136.4 (C-1 Ar), 143.3 (C-4), 148.7 (C-9), 155.3 (C-6), 161.1 (C-2); FAB-MS m/z: 253 $[M+H]^+$(matrix: mixture of dithiothreitol and dithioerythritol 3:1).

6-Phenethoxycoumarin (**2m**), colorless crystals, mp. 94°C (82% yield): ^{1}H-NMR (600 MHz, $CDCl_3$): δ 3.12 (2H, t, *J*=6.9 Hz, H-2'), 4.20 (2H, t, *J*=6.9 Hz, H-1') 6.42 (1H, d, *J*=9.6 Hz, H-3), 6.90 (1H, d, *J*=2.8 Hz, H-5), 7.10 (1H, dd, *J*=8.9 and 2.8 Hz, H-7), 7.24–7.35 (6H, m, Ar-H and H-8), 7.62 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 35.8 (C-2'), 69.5 (C-1'), 111.0 (C-5), 117.2 (C-8), 118.0 (C-3), 119.3 (C-10), 120.1 (C-7), 126.8 (C-3 Ar and C-5 Ar), 128.7 (C-4 Ar), 129.1 (C-2 Ar and C-6 Ar), 138.0 (C-1 Ar), 143.3 (C-4), 148.6 (C-9), 155.4 (C-6), 161.1 (C-2); Anal. Calcd for $C_{17}H_{14}O_3$: C, 76.68; H, 5.30. Found: C, 76.59; H, 5.67.

6-(3-Phenylpropoxy)coumarin (**2n**), colorless crystals, mp. 84–85°C (98% yield): ^{1}H-NMR (600 MHz, $CDCl_3$): δ 2.13 (2H, quint., *J*=6.2 Hz, H-2'), 2.83 (2H, t, *J*=7.6 Hz, H-3'), 3.98 (2H, t, *J*=6.2 Hz, H-1'), 6.42 (1H, d, *J*=9.6 Hz, H-3), 6.88 (1H, d, *J*=2.8 Hz, H-5), 7.10 (1H, dd, *J*=9.6 and 2.8 Hz, H-7), 7.20–7.31 (6H, m, Ar-H and H-8), 7.63 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 30.8 (C-2'), 32.2 (C-3'), 67.7 (C-1'), 110.9 (C-5), 117.2 (C-8), 118.0 (C-3), 119.3 (C-10), 120.1 (C-7), 126.2 (C-3 Ar and C-5 Ar), 128.61 (C-4 Ar), 128.64 (C-2 Ar and C-6 Ar), 141.3 (C-1 Ar), 143.4 (C-4), 148.5 (C-9), 155.6 (C-6), 161.1 (C-2); FAB-MS m/z: 281 $[M+H]^+$(matrix: mixture of dithiothreitol and dithioerythritol 3:1); Anal. Calcd for $C_{18}H_{16}O_3$: C, 77.12; H, 5.75. Found: C, 77.24; H, 6.09.

6-(4-Methoxybenzyloxy)coumarin (**2o**), colorless crystals, mp. 165°C (91% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.82 (3H, s, CH_3), 5.01 (2H, s, H-1'), 6.41 (1H, d, *J*=9.6 Hz, H-3), 6.92 (2H, d, *J*=9.0 Hz, H-Ar), 6.98 (1H, d, *J*=2.7 Hz, H-5), 7.16 (1H, dd, *J*=8.9 and 2.7 Hz, H-7), 7.25 (1H, d, *J*=8.9 Hz, H-8), 7.35 (2H, d, *J*=9.0 Hz, H-Ar), 7.63 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 55.4 (CH_3), 70.7 (C-1'), 111.5 (C-5), 114.2 (C-3 Ar and C-5 Ar), 117.1 (C-3), 118.0 (C-8), 119.3 (C-10), 120.4 (C-7), 128.4 (C-1 Ar), 129.4 (C-2 Ar and C-6 Ar), 143.3 (C-4), 148.7 (C-9), 155.4 (C-6), 159.8 (C-4 Ar), 161.1 (C-2); FAB-MS m/z: 283 $[M+H]^+$(matrix: m-nitrobenzyl alcohol);

Anal. Calcd for $C_{17}H_{14}O_4$: C, 72.33; H, 5.00. Found: C, 72.02; H, 5.08.

6-(3,5-Dimethoxybenzyloxy)coumarin (**2p**), white powder, mp. 163°C (78% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.80 (6H, s, 2(CH_3)), 5.04 (2H, s, H-1'), 6.42 (2H, d, *J*=9.4 Hz H-3 and H-Ar), 6.57 (2H, d, *J*=2.3 Hz, H-Ar), 6.97 (1H, d, *J*=2.7 Hz, H-5), 7.17 (1H, dd, *J*=9.0 and 2.7 Hz, H-7), 7.26 (1H, d, *J*=9.0 Hz, H-8), 7.63 (1H, d, *J*= 9.4 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 55.5 ($2CH_3$), 70.8 (C-1'), 100.0 (C-4 Ar), 105.3 (C-2 and C-6 Ar), 111.6 (C-5), 117.2 (C-8), 118.0 (C-3), 119.3 (C-10), 120.3 (C-7), 138.8 (C-1 Ar), 143.3 (C-4), 148.8 (C-9), 155.2 (C-6), 161.1 (C-2), 161.3 (C-3 Ar and C-5 Ar).

6-(4-Nitrophenoxy)coumarin (**2q**), pale yellow crystals, mp. 207–208°C (62% yield): ^{1}H-NMR (600 MHz, DMSO-d6): δ 6.59 (1H, d, *J*=9.6 Hz, H-3), 7.20 (2H, dd, *J*=7.0 and 2.0 Hz, Ar-H), 7.49 (1H, dd, *J*=8.9 and 2.7 Hz, H-7), 7.55 (1H, d, *J*=8.9 Hz, H-8), 7.63 (1H, d, *J*=2.7 Hz, H-5), 8.06 (1H, d, *J*=9.6 Hz, H-4), 8.27 (2H, dd, *J*=7.0 and 2.0 Hz Ar-H); ^{13}C-NMR (150 MHz, DMSO-d6): δ 117.2 (C-3), 117.5 (C-2 Ar and C-6 Ar), 118.5 (C-8), 119.5 (C-5), 120.1 (C-10), 124.6 (C-7), 126.3 (C-3 Ar and C-5 Ar), 142.5 (C-4 Ar), 143.6 (C-4), 150.3 (C-9), 150.8 (C-6), 159.8 (C-2), 162.8 (C-1 Ar); Anal. Calcd for $C_{15}H_9NO_5$: C, 63.61; H, 3.20, N, 4.95. Found: C, 63.63; H, 3.40; N, 4.55.

6-Cinnamoyloxycoumarin (**2r**) white powder, mp. 153.5°C (93% yield): ^{1}H-NMR (600 MHz, $CDCl_3$): δ 6.48 (1H, d, *J*=8.9 Hz, H-3), 6.64 (1H, d, *J*=15.8 Hz, H-2'), 7.34 (1H, d, *J*=2.7 Hz, H-5), 7.35 (1H, dd, *J*=8.7 and 2.3 Hz, H-7), 7.39 (1H, d, *J*=8.7 Hz, H-8), 7.44–7.45 (3H, m, Ar-H), 7.60–7.62 (2H, m, Ar-H), 7.69 (1H, d, *J*=8.9 Hz, H-4), 7.90 (1H, d, *J*=15.8 Hz, H-3'); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 116.6 (C-2'), 117.6 (C-3), 118.1 (C-8), 119.4 (C-10), 120.3 (C-7), 125.6 (C-5), 128.5 (C-2 Ar and C-6 Ar), 129.2 (C-3 Ar and C-5 Ar), 131.2 (C-4 Ar), 134.0 (C-1 Ar), 143.0 (C-4), 146.9 (C-9), 147.6 (C-3'), 151.6 (C-6), 160.6 (C-2), 165.4 (C-1'); Anal. Calcd for $C_{18}H_{12}O_4$: C, 73.97; H, 4.14. Found: C, 73.92; H, 4.46.

6-*(Allyloxy)coumarin* (**2s**) pale yellow crystals, mp. 86°C (93% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.54–4.58 (2H, m, H-1'), 5.33 (1H, ddd, *J*=9.0 and 1.4 Hz, H-3a'), 5.43 (1H, ddd, *J*=17.6 and 1.4 Hz, H-3b'), 5.99–6.20 (1H, m, H-2'), 6.42 (1H, d, *J*=9.8 Hz, H-3), 6.93 (1H, d, *J*=2.7 Hz, H-5), 7.14 (1H, dd, *J*=9.4 and 3.2 Hz, H-7), 7.29 (1H, d, *J*=9.4 Hz, H-8), 7.65 (1H, d, *J*=9.4 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 69.6 (C-1'), 111.3 (C-5), 117.2 (C-3), 118.0 (C-8), 118.3 (C-7), 119.3 (C-10), 120.2 (C-3'), 132.8 (C-2'), 143.3 (C-4), 148.6 (C-9), 155.1 (C-6), 161.1 (C-2); FAB-MS m/z: 203 $[M+H]^+$ (matrix: m-nitrobenzyl alcohol).

(E)-6-(2-Butenyloxy)coumarin (**2t**) white powder, mp. 119°C (88% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.78 (3H, dd, *J*=6.7 and 1.3 Hz, H-4'), 4.48 (2H, dd, *J*=5.8 and 1.3 Hz, H-1'), 5.66–5.78 (1H, m, H-2'), 5.83–5.95 (1H, m, H-3'), 6.42 (1H, d, *J*=9.4 Hz, H-3), 6.92 (1H, d, *J*=2.8 Hz, H-5), 7.13 (1H, dd, *J*=9.2 and 2.8 Hz, H-7), 7.26 (1H, d, *J*=9.2 Hz, H-8), 7.64 (1H, d, *J*=9.4 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 18.0 (C-4'), 69.5 (C-1'), 111.2 (C-5), 117.2 (C-3), 118.0 (C-8), 119.3 (C-10), 120.2 (C-7), 125.6 (C-3'), 131.3 (C-2'), 143.4 (C-4), 148.6 (C-9), 155.3 (C-6), 161.2 (C-2); FAB-MS m/z: 217 $[M+H]^+$(matrix: m-nitrobenzyl alcohol); HRFABMS m/z: 217.0874 $[M+H]^+$ (calcd for $C_{13}H_{13}O_3$, 217.0865, matrix: PEG 400); Anal. Calcd for $C_{13}H_{12}O_3$: C, 72.21; H, 5.59. Found: C, 72.28; H, 5.85.

6-(2-Butynyloxy)coumarin (**2u**) colorless crystals, mp. 138°C (92% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.87 (3H, t, *J*=2.3 Hz, H-4'), 4.69 (2H, q, *J*=2.3 Hz, H-1'), 6.43 (1H, d, *J*=9.6 Hz, H-3), 7.01 (1H, d, *J*=2.8 Hz, H-5), 7.17 (1H, dd, *J*=9.2 and 2.8 Hz, H-7), 7.28 (1H, d, *J*=9.2 Hz, H-8), 7.67 (1H, d, *J*=9.6 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 3.8 (C-4'), 57.2 (C-1'), 73.6 (C-3'), 84.6 (C-2'), 111.7 (C-5), 117.3 (C-8), 118.0 (C-3), 119.3 (C-10), 120.3 (C-7), 143.4 (C-4), 148.9 (C-9), 154.3 (C-6), 161.1 (C-2); FAB-MS m/z: 215 $[M+H]^+$(matrix: m-nitrobenzyl alcohol); HRFABMS m/z: 215.0717 $[M+H]^+$ (calcd for $C_{13}H_{11}O_3$, 215.0708, matrix: PEG 400); Anal. Calcd for $C_{13}H_{10}O_3$: C, 72.89; H, 4.71. Found: C, 72.86; H, 4.94.

6-(2-Pentynyloxy)coumarin (**2v**) colorless crystals, mp. 116°C (85% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.14 (3H, t, *J*=7.6 Hz, H-5'), 2.23 (2H, qt, *J*=7.6 and 2.2 Hz, H-4'), 4.71 (2H, t, *J*=2.2 Hz , H-1'), 6.43 (1H, d, *J*=9.4 Hz, H-3), 7.03 (1H, d, *J*=2.7 Hz, H-5), 7.17 (1H, dd, *J*=9.2 and 2.7 Hz, H-7), 7.27 (1H, d, *J*=9.2 Hz, H-8), 7.67 (1H, d, *J*=9.4 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 12.6 (C-5'), 13.7 (C-4'), 57.2 (C-1'), 73.7 (C-2'), 90.4 (C-3'), 111.8 (C-5), 117.2 (C-3), 117.9 (C-8), 119.2 (C-10), 120.3 (C-7), 143.3 (C-4), 148.9 (C-9), 154.3 (C-6), 161.0 (C-2); FAB-MS m/z: 229 $[M+H]^+$(matrix: m-nitrobenzyl alcohol); HRFABMS m/z: 229.0893 $[M+H]^+$ (calcd for $C_{14}H_{13}O_3$, 229.0865, matrix: PEG 400); Anal. Calcd for $C_{14}H_{12}O_3$: C, 73.67; H, 5.30. Found: C, 73.43; H, 5.31.

6-(2-Octynyloxy)coumarin (**2w**) pale yellow crystals, mp. 47°C (89% yield): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.86 (3H, t, *J*=7.2 Hz, H-8'), 1.21–1.37 (4H, m, H-7' and H-6'), 1.43–1.54 (2H, m, H-5'), 2.16–2.34 (2H, m, H-4'), 4.72 (2H, t, *J*=1.8 Hz H-1'), 6.43 (1H, d, *J*=9.4 Hz, H-3), 7.03 (1H, d, *J*=2.7 Hz, H-5), 7.18 (1H, dd, *J*=9.0 and 2.7 Hz, H-7), 7.27 (1H, d, *J*=9.0 Hz, H-8), 7.67 (1H, d, *J*=9.4 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 14.1 (C-8'), 18.8 (C-4'), 22.2 (C-7'), 28.2 (C-5'), 31.1 (C-6'), 57.2 (C-1'), 74.4 (C-2'), 89.3 (C-3'), 111.9 (C-5), 117.2 (C-3), 117.9 (C-8), 119.2 (C-10), 120.4 (C-7), 143.3 (C-4), 148.9 (C-9), 154.3 (C-6), 161.1 (C-2); FAB-MS m/z: 271 $[M+H]^+$; Anal. Calcd for $C_{17}H_{18}O_3$: C, 75.53; H, 6.71. Found: C, 75.29; H, 7.11.

Termites Workers and soldiers of *C. formosanus* Shiraki were obtained from a laboratory colony maintained at 28°C±2 and >85% Relative Humidity (RH) at the Deterioration Organisms Laboratory of the Research Institute for Sustainable Humanosphere (RISH), Kyoto University, Japan.

Termiticidal and Antifeedant Tests A no-choice test was employed for evaluating termiticidal and antifeedant activities (Adfa et al., 2010b). Each solution of the 6-substituted coumarin analogs **2a**–**2w** (5 μmol and 10 μmol) was dissolved in 0.05 ml chloroform and applied to paper discs (1.5 mm thick, 8 mm diam and average mass 30 mg, Advantec, Tokyo-Japan); the paper discs were dried overnight in a vacuum desiccator. Subsequently, each disc was placed on a plastic spacer in an acrylic resin cylinder with a plaster bottom (80 mm diam×60 mm in height), and 33 active termites (30 workers and three soldiers) were added to each cylinder. The cylinders were placed on a cotton sheet in a plastic tray and kept in a dark incubator at 28°C±2 and 80%±5 RH. Paper discs treated only with chloroform were used as a control. Three replications were performed for each sample.

Termite Bioassay Data Analysis The number of dead termites was counted daily for 2 weeks. Paper disc consumption by the termites was measured at the end of the test. Mass loss of the paper discs was determined by subtracting the vacuum-dried weight at the end of the test from that before the start of the test. Cumulative daily mortality and consumption (mean and standard deviation) were calculated from the three replicates of each treatment.

Statistical Analysis Tukey's multiple comparison test was used to evaluate differences in percent mortality and mass loss in antitermite activities. Results with $P<0.05$ were considered significant. Calculations were performed using statistical software (XLSTAT, Addinsoft, New York, USA).

Results and Discussion

Synthesis of 6-Alkoxycoumarins and Related Analogs 6-Methoxycoumarin (**2a**) was synthesized according to a previous report (Adfa et al., 2010b). The remaining 22 coumarin analogs (**2b**–**2w**) were synthesized from the commercially available 6-hydroxycoumarin (**1**) by treatment with various alkyl/aryl/acyl halides and potassium carbonate in DMF using different reaction conditions (Scheme 1, Table 1). The corresponding products were obtained in good to high yields (62–99%), with the exception of product **2k** that was acquired in moderate yield (41%). The preparation of **2r** failed under the standard conditions (K_2CO_3, DMF, 80°C). However, when Et_3N and DMAP were used instead of K_2CO_3, **2r** was obtained in 93% yield (Table 1). Of the analogs prepared and tested, 20 compounds are novel. Exceptions are 6-benzyloxycoumarin (**2l**) and 6-allyloxycoumarin (**2s**), which were reported earlier by Shah and Trivedi (1975), and Durst et al. (2007). All compounds were characterized by ^{1}H-NMR, ^{13}C-NMR, MS, and elemental analysis; additionally, the structure of compound **2l** was determined by X-ray crystallography (Adfa et al., 2010a). To summarize, we synthesized 23 compounds and tested them for antifeedant and termiticidal activities against *C. formosanus*.

Termiticidal Activity of 6-Alkoxycoumarins and Related Analogs Against C. formosanus Shiraki The mortality of *C. formosanus* exposed to 6-alkoxycoumarins and related analogs (**2a**–**2w**) was determined daily for two weeks. The results are summarized in Figs. 1 and 2. Among the 23 coumarin derivatives, eight compounds (**2a**, **2f**, **2m**, **2o**, **2s**, **2t**, **2u**, **2v**, and **2w**) demonstrated termite mortalities that were significantly higher than that of the control at concentrations of 5 and 10 μmol (Table 2). Compound (**2u**) 6-(2-Butynyloxy)coumarin showed the highest termite mortality (58.59%) at a concentration of 5 μmol (Fig. 1). At a concentration of 10 μmol, 6-(2-pentynyloxy)coumarin (**2v**) exhibited the strongest mortality (74.75%), followed consecutively by 6-(2-butynyloxy)coumarin (**2u**) (52.53%), 6-(2-octynyloxy)coumarin (**2w**) (44.44%), and 6-

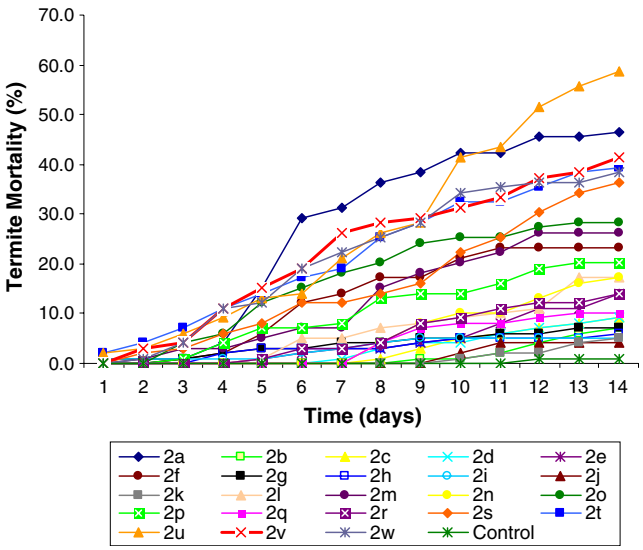


Fig. 1 Termite mortalities (%) of 6-alkoxycoumarins and related analogs (**2a**–**2w**) against *Coptotermes formosanus* Shiraki in no-choice test at concentration of 5 μmol. Each experiment was performed in triplicate. Thirty-three termites were used per replication and the data was averaged (N=3)

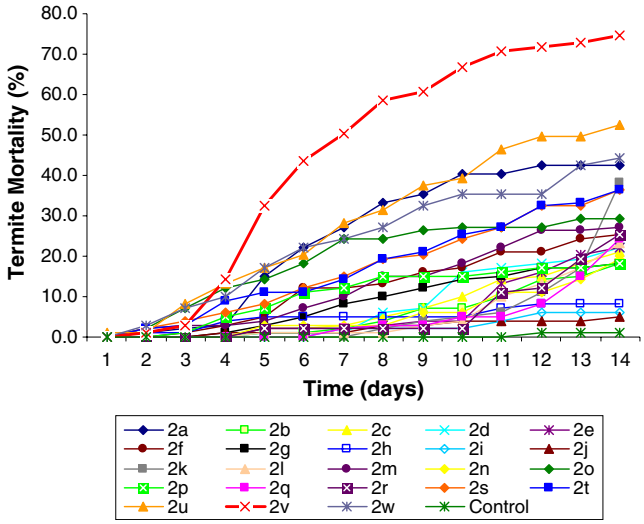


Fig. 2 Termite mortalities (%) of 6-alkoxycoumarins and related analogs (**2a**–**2w**) against *Coptotermes formosanus* Shiraki in no-choice test at concentration of 10 μmol. Each experiment was performed in triplicate. Thirty-three termites were used per replication and the data was averaged (N=3)

methoxycoumarin (**2a**) (42.42%). Termite mortality with **2v** at a concentration of 10 μmol was 1.8 times higher than that at 5 μmol, while the mortalities of **2a** and **2u** at 10 μmol showed a slight decrease compared to that at 5 μmol concentration, but the difference was not statistically significant. However, different patterns of mortality were observed; the mortality of **2v** at 10 μmol started to increase sharply after four days, while the mortalities of **2a** and **2u** showed a more steady increase (Fig. 2).

Four additional analogs, 6-Cyclohexyloxycoumarin (**2k**), 6-benzyloxycoumarin (**2l**), 6-(4-nitrophenoxy)coumarin (**2q**), and 6-cinnamoyloxycoumarin (**2r**) yielded termite mortalities that were significantly higher than that of the control at a concentration of 10 μmol. Interestingly, termite mortality with **2k** at 10 μmol increased sharply after 11 days and showed an eight-fold gain compared with the 5 μmol concentration (Fig. 2). The least active analogs were 6-ethoxycoumarin (**2b**), 6-propoxycoumarin (**2c**), 6-isopropoxycoumarin (**2d**), 6-butoxycoumarin (**2e**), 6-octyloxycoumarin (**2g**), 6-dodecyloxycoumarin (**2h**), 6-

Table 2 Statistical analysis of termiticidal activity of 6-alkoxycoumarin and related analogs

Compound	Mortalities of termite (%) after 2 weeks	
	[5 μmol]	[10 μmol]
6-Methoxycoumarin (**2a**)	$46.46\pm7.6^{b, c, d}$	$42.42\pm8.0^{b, c, d, e, f}$
6-Ethoxycoumarin (**2b**)	$7.07\pm4.6^{k, l, m, n, o}$	$18.18\pm3.0^{h, i, j, k, l, m, n, o}$
6-Propoxycoumarin (**2c**)	$9.09\pm3.0^{j, k, l, m, n, o}$	$21.21\pm3.0^{f, g, h, i, j, k, l, m, n, o}$
6-Isopropoxycoumarin (**2d**)	$9.09\pm3.0^{j, k, l, m, n, o}$	$22.22\pm4.6^{f, g, h, i, j, k, l, m, n, o}$
6-Butoxycoumarin (**2e**)	$14.14\pm4.6^{j, k, l, m, n, o}$	$22.22\pm4.6^{f, g, h, i, j, k, l, m, n, o}$
6-Isobutoxycoumarin (**2f**)	$23.23\pm6.3^{e, f, g, h, i, j, k, l, m, n}$	$25.25\pm7.0^{d, e, f, g, h, i, j, k, l, m, n}$
6-Octyloxycoumarin (**2g**)	$7.07\pm4.6^{k, l, m, n, o}$	$18.18\pm6.1^{h, i, j, k, l, m, n, o}$
6-Dodecyloxycoumarin (**2h**)	$6.06\pm3.0^{l, m, n, o}$	$8.08\pm4.6^{j, k, l, m, n, o}$
6-Hexadecyloxycoumarin (**2i**)	$5.05\pm3.5^{m, n, o}$	$6.06\pm3.0^{l, m, n, o}$
6-Octadecyloxycoumarin (**2j**)	$4.04\pm1.7^{n, o}$	$5.05\pm6.3^{m, n, o}$
6-Cyclohexyloxycoumarin (**2k**)	$5.05\pm3.5^{m, n, o}$	$38.38\pm7.6^{b, c, d, e, f, g, h, i}$
6-Benzyloxycoumarin (**2l**)	$17.17\pm4.6^{i, j, k, l, m, n, o}$	$23.23\pm4.6^{e, f, g, h, i, j, k, l, m, n}$
6-Phenethoxycoumarin (**2m**)	$26.26\pm3.5^{d, e, f, g, h, i, j, k, l, m}$	$27.27\pm3.0^{d, e, f, g, h, i, j, k, l}$
6-(3-Phenylpropoxy)coumarin (**2n**)	$17.17\pm7.6^{i, j, k, l, m, n, o}$	$19.19\pm4.6^{h, i, j, k, l, m, n, o}$
6-(4-Methoxybenzyloxy)coumarin (**2o**)	$28.28\pm1.7^{d, e, f, g, h, i, j, k}$	$29.29\pm3.5^{d, e, f, g, h, i, j}$
6-(3,5-Dimethoxybenzyloxy)coumarin (**2p**)	$20.20\pm1.7^{g, h, i, j, k, l, m, n, o}$	$18.18\pm6.1^{h, i, j, k, l, m, n, o}$
6-(4-Nitrophenoxy)coumarin (**2q**)	$10.10\pm1.7^{j, k, l, m, n, o}$	$24.24\pm13.9^{e, f, g, h, i, j, k, l, m, n}$
6-Cinnamoyloxycoumarin (**2r**)	$14.14\pm4.6^{j, k, l, m, n, o}$	$25.25\pm12.6^{d, e, f, g, h, i, j, k, l, m, n}$
6-(Allyloxy)coumarin (**2s**)	$36.36\pm10.5^{c, d, e, f, g, h, i}$	$36.36\pm8.0^{c, d, e, f, g, h, i}$
(*E*)-6-(2-Butenyloxy)coumarin (**2t**)	$39.39\pm9.1^{b, c, d, e, f, g, h}$	$36.36\pm3.0^{c, d, e, f, g, h, i}$
6-(2-Butynyloxy)coumarin (**2u**)	$58.59\pm21.3^{a, b}$	$52.53\pm4.6^{b, c}$
6-(2-Pentynyloxy)coumarin (**2v**)	$41.41\pm4.6^{b, c, d, e, f, g}$	74.75 ± 7.6^{a}
6-(2-Octynyloxy)coumarin (**2w**)	$38.38\pm9.7^{b, c, d, e, f, g, h, i}$	$44.44\pm3.5^{b, c, d, e}$
Control	0.01 ± 1.7^{o}	

Data are given as mean ± standard deviation of triplicate tests (N=3). Numbers followed by different letters (a–o) are significantly different at the level of $P<0.05$ according to Tukey's multiple comparison test

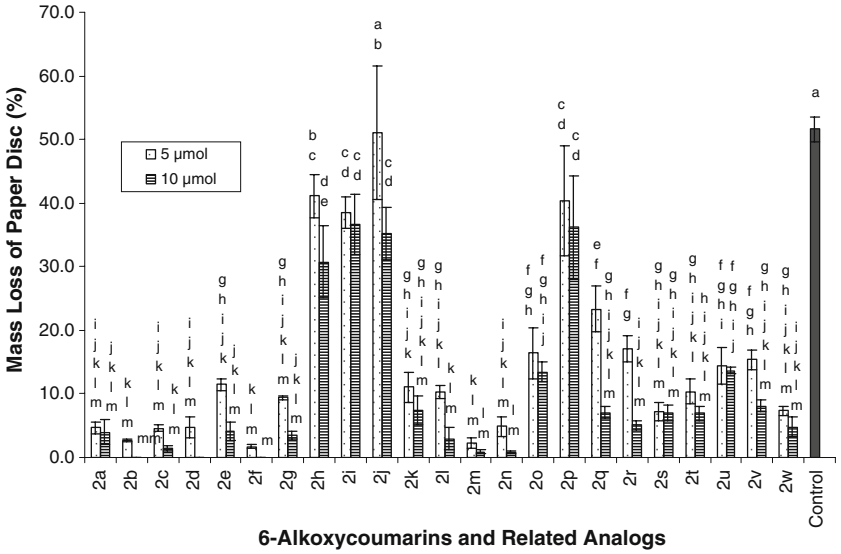


Fig. 3 Paper disc consumption by *Coptotermes formosanus* Shiraki after 14 d exposure to 6-alkoxycoumarins and related analogs (**2a**–**2w**) in no-choice test. Each experiment was performed in triplicate. Thirty-three termites were used per replication and the data were averaged (N=3). *Different letters* (a–m) indicate significant difference (Tukey's multiple comparison test; P<0.05). *Error bars* represent standard deviations

hexadecyloxycoumarin (**2i**), 6-octadecyloxycoumarin (**2j**), 6-(3-phenylpropoxy)coumarin (**2n**), and 6-(3,5-dimethoxybenzyloxy)coumarin (**2p**), which demonstrated no significant difference from the control at either concentration. In general, the presence of alkynoxy and alkenoxy groups was found to be important for the termiticidal activity. Furthermore, the existence of alkoxy groups with a longer alkyl chain tended to decrease the activity.

Antifeedant Activity of 6-Alkoxycoumarins and Related Analogs Against C. formosanus Shiraki Paper disc consumption by the termites after 14 days exposure to the 6-alkoxycoumarins and related analogs (**2a**–**2w**) was compared to that of the control, and the average mass losses for the 23 compounds are shown in Fig. 3. At both concentrations, all analogs showed antifeedant activity, with the exception of 6-octadecyloxycoumarin (**2j**) that had a 51.12% mass loss of the paper disc at a concentration of 5 µmol, essentially the same as that of the control. Considering both concentrations, this compound exhibited the lowest antifeedant activity with a higher average mass loss than that exhibited by other compounds. Compound (**2f**) 6-isobutoxycoumarin elicited the lowest average mass loss at a concentration of 5 µmol (1.65%), showing a significantly greater reduction in feeding activity compared to the control (51.61%), but no significant difference was observed between **2f** and 13 other compounds (**2a** 4.69%, **2b** 2.71%, **2c** 4.62%, **2d** 4.72%, **2e** 11.47%, **2g** 9.37%, **2k** 11.10%, **2l** 10.25%, **2m** 2.28%, **2n** 4.86%, **2s** 7.27%, **2t** 10.35%, and **2w** 7.35%) at the same concentration (Tukey's multiple comparison test). At a concentration of 10 µmol, 6-ethoxycoumarin (**2b**), 6-isopropoxycoumarin (**2d**), and 6-isobutoxycoumarin (**2f**) exhibited the strongest antifeedant activity with no mass loss (0.00%) of the paper disc. However, in the statistical analysis, no significant difference was observed between these analogs and 14 other compounds (**2a** 3.98%, **2c** 1.48%, **2e** 4.02%, **2g** 3.51%, **2k** 7.48%, **2l** 2.98%, **2m** 0.91%, **2n** 0.80%, **2q** 7.09%, **2r** 5.09%, **2s** 7.03%, **2t** 6.90%, **2v** 8.10%, and **2w** 4.81%). The results suggested that 6-alkoxycoumarins having a short alkyl chain (methyl–octyl) showed high antifeedant activity, while increasing the length of the alkyl chain (dodecyl, hexadecyl, and octadecyl) led to a sharp decrease in activity. The presence of arylalkoxy groups was important for antifeedant activity, but methoxy substitution on the benzene ring was a disadvantage. In addition, the existence of alkynoxy and alkenoxy led to analogs with good activity.

In summary, we synthesized and tested a number of 6-alkoxycoumarins and related analogs, and showed effective termiticidal and antifeedant activities against *C. formosanus*. SAR studies summarized the key features that are important for activities and demonstrated the potential of 6-alkoxycoumarin derivatives to be used as termite control agents.

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Ecosystem, Location, and Climate Effects on Foliar Secondary Metabolites of Lodgepole Pine Populations from Central British Columbia

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Abstract Lodgepole pines, *Pinus contorta* Douglas ex Louden var. *latifolia* Engelm. ex S. Watson, are encountering increased abiotic stress and pest activity due to recent increases in temperature and changes in precipitation throughout their range. This tree species counters these threats by producing secondary metabolites, including phenolics and terpenoids. We examined foliar levels of lignin, soluble phenolics, monoterpenoids, sesquiterpenoids, and diterpenoids in 12 stands in British Columbia, Canada. We used these data to assess associations among foliar secondary metabolite levels and ecosystem, geographic, and climatic variables. Regressions were also performed to observe which combinations of variables best explained secondary metabolite variance. Stands of *P. c. latifolia* in the Coastal Western Hemlock and Interior Cedar/Hemlock biogeoclimatic zones had consistently greater foliar levels of almost all measured secondary metabolites than did other stands. Lignin was present in greater amounts in Boreal White/Black Spruce ecosystem (i.e., northern) stands than in southern stands, suggesting a role for this metabolite in pine survival in the boreal forest. Attempts to develop regression models with geographic and climatic variables to explain foliar secondary metabolite levels resulted in multiple models with similar predictive capability. Since foliar secondary metabolite levels appeared to vary most between stand ecosystem types and not as much due to geographic and climatic variables, metabolic profiles appeared best matched to the stress levels within local environments. It is unknown if differences in secondary metabolite levels are the result of genetic adaptation or phenotypic plasticity, but results from this and other studies suggest that both are important. These results are interpreted in light of ongoing efforts to assist in the migration of certain populations of *P. c. latifolia* northward in an effort to counter predicted effects of climate change.

Key Words Climate change · Gas chromatography–mass spectrometry (GC-MS) · Lignin · Lodgepole pine · Monoterpenoid · Phenolics · *Pinus contorta latifolia* · Sesquiterpenoid · Diterpenoid

Introduction

Lodgepole pine, *Pinus contorta* Douglas ex Louden var. *latifolia* Engelm. ex S. Watson (http://www.itis.gov/), is a valuable and widespread species found throughout western North America, where it is widely planted as a part of sustainable forestry practice (Wu et al., 2005; Ying and Yanchuk, 2006). However, in recent years many plantations of *P. c. latifolia* throughout its range have been severely impacted by generally warmer temperatures (especially in winter) and localized changes in precipitation (Folke et al., 2004). In addition to climate adversely affecting tree physiology, and in turn productivity, there has been an increase in pest activity, including widespread mortality caused by the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae) (Walther et

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al., 2002; Kurz et al., 2008; Bentz et al., 2010) and regionally important outbreaks of *Dothistroma* needle cast [causal agent: *Mycosphaerella pini* Rost. in Munk; anamorph *Dothistroma septospora* (Dorog.) Morelet] (Ascomycota: Mycosphaerellaceae) (Woods et al., 2005). It is hypothesized that in the future, climate change will continue and even intensify, which will further impact pine growth and result in more frequent pine pest and disease outbreaks (Coakley et al., 1999; Logan et al., 2003; Desprez-Loustau et al., 2007; Bentz et al., 2010).

To counter the potential negative effects of climate change, efforts have begun to move individuals from particular pine populations to areas whose future climates are predicted to be optimal for their growth in a process called assisted migration (O'Neill et al., 2008). Assisted migration involves moving pine populations to higher latitudinal or elevational sites because it is hypothesized that certain populations of trees are better-suited to areas which, although also warming, will remain cooler overall (Coakley et al., 1999; O'Neill et al., 2008; Marris, 2009).

It remains unclear what impacts the assisted migration of certain populations of *P. c. latifolia* into new areas will have on their ability to thrive in the near- and long-terms (Marris, 2009). Although attempts have been made to find suitable locations to minimize climate change effects on *P. c. latifolia* physiology (Cumming and Burton, 1996; Hamann and Wang, 2006; O'Neill et al., 2008), our understanding of this process is in its infancy (Wu et al., 2005; Wallis et al., 2010). Populations of *P. c. latifolia* that originated from different ecosystems, or that were far apart geographically, have been found to vary not only in growth and reproduction, but also in their susceptibility to diseases (Rehfeldt et al., 1999; Wu et al., 2005; Heineman et al., 2010; Wallis et al., 2010). Such distinct populations also were observed to vary in foliar secondary metabolite (SM) levels, at least when they were planted together at one seed orchard site (Wallis et al., 2010). Overall differences in the ability of populations of *P. c. latifolia* to synthesize SMs, such as phenolics and terpenoids, are likely to have led to observed variations in growth rates and susceptibility to pests, because such compounds have a variety of roles including photoprotection, relieving oxidative stress, reducing water stress, and protection from insect and pathogen pests (Keeling and Bohlmann, 2006; Seybold et al., 2006; Whitzell and Martin, 2008).

Populations of *P. c. latifolia* from lower latitudes and from warmer, wetter stands will need increased protection from biotic stresses, and therefore the possession of greater pest defense-associated SM levels (both phenolics and terpenoids) will confer fitness advantages (e.g., Franceschi et al., 2005; Wallis et al., 2008; Adams et al., 2009). In contrast, *P. c. latifolia* from northern, colder, and drier locales will gain a fitness advantage by putting more resources into primary metabolites, presumably at the cost of producing certain SMs, in order to deal with a shorter growing season and to outcompete neighboring plants for scarce resources (Siska et al., 2002; Andrew and Hughes, 2005; Adams et al., 2009).

However, other SM classes might play larger roles in conferring resistance to abiotic stresses than in their abilities to combat pests; for instance, *P. c. latifolia* from boreal forest locations may possess greater cell-wall thickening phenolics such as lignin that could confer fitness advantages in overcoming abiotic stresses such as extreme cold (Smallwood and Bowles, 2002; Wei et al., 2006). Levels of other SMs, in particularly flavonols, are positively associated with latitude presumably because they are involved in resisting abiotic stresses (Lätti et al., 2008; Stark et al., 2008; Martz et al., 2009, 2010). Day lengths, lowered temperatures, and UV exposure are all implicated in the relationship between latitude and levels of certain SMs (reviewed by Jaakola and Hohtola, 2010).

Therefore, levels of biotic and abiotic stresses imparted on populations can positively or negatively affect distinct groups of SMs, depending on the class of the compounds and the particular roles that they play in resistance to stress. The aim of our study was to quantify the differences in foliar SM levels among stands of naturally regenerating *P. c. latifolia* that should be exposed to a variety of abiotic and biotic stresses. We examined variations in SM levels—including total soluble phenolics, lignin, and terpenoids—in the foliage from 12 distinct stands of *P. c. latifolia*. Ecological, geographical, and climatic differences among these sites were examined to observe which variables might influence the levels of these compound classes. We hypothesized that associations among SM classes and ecological, geographic, or climatic variables will be due, in part, to the particular roles they play in countering abiotic or biotic stresses.

Methods and Materials

Stand Descriptions and Sampling A total of 12 mature stands of *P. c. latifolia* of natural origin were assessed across central British Columbia (Fig. 1). These stands were from a variety of habitats and climates comprising five distinct ecosystems as delineated by British Columbia's biogeoclimatic (BEC) zone system. They included the very cold boreal white and black spruce (BWBS) zone; the very wet Coastal Western Hemlock (CWH) zone; the high elevation Englemann Spruce/Subalpine Fir (ESSF) zone; the very wet Interior Cedar/Hemlock zone (ICH); and the widespread Sub-boreal Spruce (SBS) zone (Meidinger and Pojar, 1991). Climate data for each sampling site [mean summer (June through August), mean winter (December through February) and mean annual temperatures; and

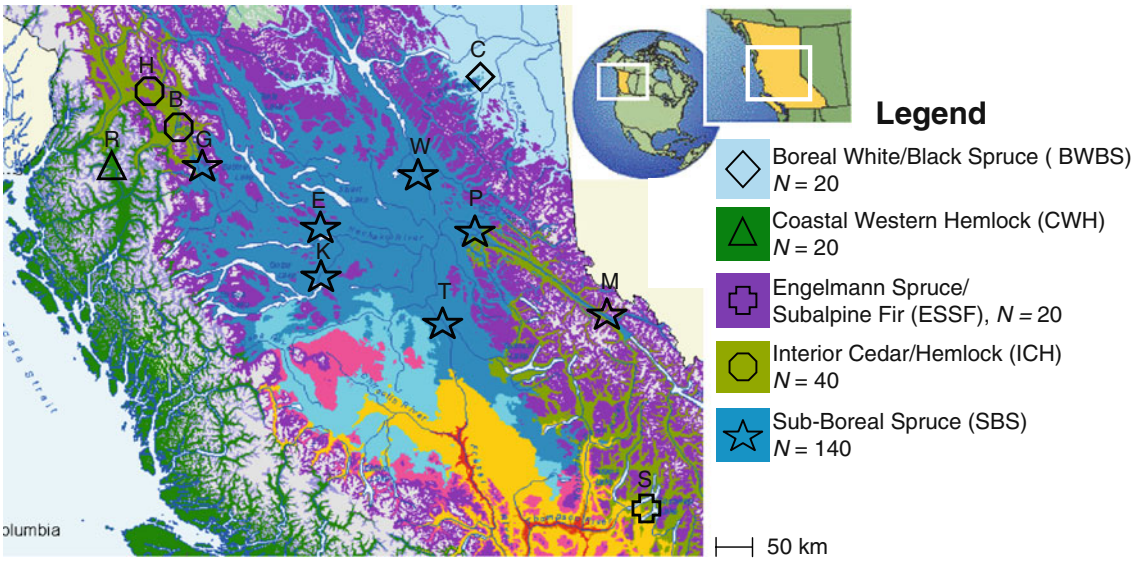


Fig. 1 Locations of populations of *Pinus contorta latifolia* in British Columbia, Canada identified by letter corresponding to site names in Table 1; and their British Columbia biogeoclimatic (BEC) classification and sample size. Map courtesy of the BC Ministry of Forests and Range (http://www.for.gov.bc.ca/hre/becweb/)

mean summer (June through August) and mean annual precipitation levels] were determined by using the Climate BC program, based upon measured temperature and precipitation data from 1961 to 1990 (Wang et al., 2006) (Table 1). Sampling occurred between June 15, and July 15, 2009, and at each stand the trees were in the same phenological stage (near the completion of needle expansion from newly-formed candles). At each site, approximately 5 g of apparently insect- and disease-free, second-year needles (i.e., those that were formed in 2008) were collected from three

Table 1 Coordinates and site characteristics for the *Pinus contorta latifolia* stands assessed for foliar secondary metabolites

Site Name	Elev. (m)	Zone	Easting	Northing	Biogeoclimatic (BEC) Zone	Mean Summer Temperature (°C)	Mean Winter Temperature (°C)	Mean Annual Temperature (°C)	Mean Summer Prec.(cm)	Mean Annual Prec. (cm)
Chetwynd (C)	925	10U	587,673	6,179,135	Boreal White/ Black Spruce (BWBS)	13.6	−8.3	1.7	33.9	57.8
Rosswood (R)	185	9U	514,695	6,075,002	Coastal Western Hemlock (CWH)	13.9	−3.9	5.1	34.8	114.1
Salmon Arm (S)	1,424	11U	327,518	5,621,723	Engelmann Spruce/ Subalpine Fir (ESSF)	13.0	−7.1	3.0	34.5	84.7
Bulkley Canyon (B)	396	9U	598,078	6,120,028	Interior Cedar Hemlock (ICH)	13.8	−6.8	3.8	26.0	58.5
Cullon Creek (H)	509	9U	564,007	6,165,179	ICH	13.4	−7.3	3.4	32.8	65.8
Burns Lake (E)	860	10U	322,846	6,013,982	Sub-boreal Spruce (SBS)	12.2	−9.0	2.0	22.2	49.7
Ganokwa Creek(G)	851	9U	631,887	6,074,792	SBS	12.5	−8.0	2.3	22.0	51.7
Kenny Dam (K)	898	10U	374,880	5,937,816	SBS	12.2	−9.1	2.1	23.6	50.6
McBride (M)	759	11U	324,779	5,882,146	SBS	14.4	−8.3	3.4	34.2	80.9
Purden (P)	729	10U	566,464	5,972,171	SBS	13.4	−8.2	3.0	34.3	78.5
Ten Mile Lake (T)	733	10U	537,587	5,880,356	SBS	14.6	−7.4	4.1	27.5	58.3
Whiskers Point (W)	687	10U	503,894	6,084,477	SBS	13.7	−9.7	2.4	26.6	75.4

randomly chosen branches from each of 20 naturally regenerating trees ranging from 15 to 25 year-old. The samples were immediately placed onto dry ice for transport to the lab, where they were stored at −20°C until processed for phytochemical analyses roughly 1 month later.

Phytochemical Analyses Needle samples were ground by mortar and pestle in liquid nitrogen, with 100 mg of ground tissue twice-extracted in 1 ml of methanol (Thermo-Fisher Scientific, Pittsburg, PA, USA) and another 100 mg of tissue twice-extracted in 1 ml of dichloromethane (methylene chloride) (Sigma-Aldrich, St. Louis, MO, USA) according to the protocols of Wallis et al. (2008). The methanol extracts were assessed for total phenolics by using the Folin-Ciocalteu reagent spectrophotometric method (with an instrument from Perkin-Elmer, Waltham, MA, USA) for quantifying soluble phenolics, and the pellet left behind following the methanol extraction was further processed to quantify total lignin (bound to the cell walls) according to the procedures of Wallis et al. (2008) with all reagents from Sigma-Aldrich. In brief, the pellets were washed once with 1 ml water, then 1 ml methanol, and finally 1 ml of petroleum ether and were allowed to dry overnight. To the pellet, 200 μl of 1 N NaOH were added, and this was left in a shaker at 40°C for 21 h, after which 200 μl of 1.5 M formic acid and 400 μl of methanol were added. The mixture was centrifuged at 10,000 x *g* for 5 min, and the supernatant removed. The pellet then was washed in 1 ml of water and re-suspended in 800 μl of 2 N HCl and 300 μl of thioglycolic (mercaptoacetic) acid heated at 86°C for 4 h. The tubes were spun down for 5 min at 10,000 x *g*, and the supernatant removed, with the pellet then twice-washed in 1 ml water. The pellet was then re-suspended in 1 ml of 0.5 M NaOH and left for 18 h on a shaker. Following centrifugation, the supernatant was transferred to a clean tube and the pellet re-suspended in 0.5 ml of 0.5 M NaOH. Following centrifugation, the supernatant was combined with that from the previous step to total a 1.5 ml of 0.5 M NaOH solution. To this, 300 μl of concentrated (12 N) HCl were added and allowed to stand at room at room temperature for 4 h. The solution was spun down for 5 min at 10,000 x *g*, and the supernatant discard. The pellet was re-suspended in 1 ml of 0.5 M NaOH, and read on a spectrophotometer (Perkin-Elmer) at 280 nm. Lignin concentrations in mg were determined by using a standard curve made from a dilution series of commercially available lignin standard from spruce (Sigma-Aldrich).

The dichloromethane extracts were analyzed for terpenoid content (monoterpenoids, sesquiterpenoids, and diterpenoids) with a Varian Model 3800 gas chromatograph with a flame-ionization detector and a split line to a Saturn 2200 Ion Trap MS-MS (Agilent, Palo Alto, CA, USA) and *n*-pentadecane (Sigma-Aldrich) as an internal standard (Wallis et al., 2010). The column was a 30 m×0.25 mm i.d.×0.25 μm film thickness Agilent Ultra 2.5% phenylmethylsiloxane capillary column; the carrier gas was helium running at a constant flow of 2.1 ml/min; the split/splitless injection port was in split mode (10:1 ratio) with the temperature set at 320°C; and the detector/transfer line temperatures were set at 320°C. The temperature program started with a hold of 2 min at 40°C; ramped at 10°C/min to 200°C; and finally ramped at 50°C/min to a final temperature of 300°C (with a 5 min final hold) (total run time of 28.20 min). All peak areas of identified terpenes were summed together with others in their class (monoterpenoids, sesquiterpenoids, or diterpenoids) to give an overall value for each terpenoid group. Compound identifications were based on retention time matching to known standards and matching to mass spectral libraries with reverse search indices higher than 850 (Wallis et al., 2008). Stereoisomers of certain compounds (e.g., α-pinene, β-pinene, and limonene) went unresolved, as this study stressed changes in total terpenoid group production (i.e., the regulation upstream of individual monoterpenes, sesquiterpenes, and diterpenes). The toxicity of terpenoids to pathogens is thought to not depend on the enantiomeric composition (Wallis et al., 2008). Putative diterpenoids, labeled in this work as unknown diterpenoids, were compounds whose matches to mass spectral libraries contained a typical diterpenoid skeleton of C_{20} and for which the top five library matches were different diterpenoids. However, for these putative diterpenoids no single compound exceeded the reverse search index threshold set at 850.

Statistical Analyses All statistical tests were performed with either SPSS ver. 17 (SPSS Inc., Chicago, IL, USA) or R ver. 2.10.0 (CRAN, Wirtschaftsuniversität Wien, Austria) at a threshold of $\alpha=0.05$. Normality was assessed by examining residual plots and histograms, and Levene's tests for variance heterogeneity were performed. Pearson's correlations were used to find associations between groups of foliar compounds. Univariate ANOVAs were used to find differences in phytochemistry between trees grown in different BEC zones. Tukey HSD tests were used to separate individual BEC zone means.

Coordinates were first converted from UTM to BC Albers, a metric that is continuous throughout British Columbia (http://geobc.gov.bc.ca) for all analyses that examined relationships between geographical variables and SM levels. Specifically, the BC Albers has an x-coordinate that represents longitude (with increasing numbers representing movement eastward) and a y-coordinate that represents latitude (with increasing numbers representing movement northward).

Pearson's correlations were used to examine potential relationships between geographic or climatic variables and

foliar SM levels. One analysis compared the mean levels of the SMs for each site with these variables, whereas another used the SM values for individual trees. The first analysis was performed to emphasize the geographic or climatic effects on a typical individual in the tree's population, whereas the latter emphasized effects on multiple individuals in the same stand of trees. This was also performed in part due to concerns over the large sample size (greater than 250 replicates overall) of SM levels potentially skewing these associations, considering that 12 estimates were made for each of the geographical and climatic variables. An additional analysis compared the levels of SMs from individual trees vs. geographic or climatic variables with data from the SBS zone only, as this zone was represented far more (7 times) than the other sites and is arguably the primary ecosystem type in which *P. c. latifolia* is found in British Columbia.

Linear regression analyses were performed to observe which combination of variables best explained the variance in foliar secondary metabolites. Using the "Rcmdr" package within R, a linear regression model for each SM group as explained by all the geographic and climatic variables was loaded. These models then underwent the "subset model selection…" application under Rcmdr's "Model" menu, which output a list of the variables included in the models that had the lowest Schwarz's Bayesian Information Criteria (BIC) values (with the lowest values representing the best models) (Schwarz, 1978). The strongest model matched that selected via Stepwise Regression Analysis based on BIC; however, the next two top models also were selected for comparison as they could not be dismissed due to their possessing similar BIC values (within ~2) to the top model (Burnham and Anderson, 2002). For additional evaluation, the Akaike Information Criteria (AIC) values also were assessed (Akaike, 1974). These regressions were performed on the mean SM levels at each site; on SM levels for all of the individual trees; and on the SM levels for all individual trees for the SBS zone alone.

Results

Secondary Metabolite Identities and Associations In this study, we assessed a total of 14 monoterpenoids (sabinene, α-pinene, camphene, β-pinene, β-myrcene, α-phellandrene, δ-3-carene, *p*-cymene, β-phellandrene, δ-2-carene, linalool, camphor, bornyl acetate, and verbanone), seven sesquiterpenoids (α-cubenene, α-copanene, aristolene, β-cadinene, τ-cadinol, germacrenol, and an unidentified sesquiterpenoid), and five diterpenoids (all of which were unable to be identified to specific compounds). We also assessed total phenolic (with Folin-Ciocalteu reagent) and total lignin content.

Foliar phenolic levels were significantly and positively associated with the foliar levels of all three groups of terpenoids; and sesquiterpenoid levels were positively associated with levels of both monoterpenoids and diterpenoids (Table 2). No other significant correlations were observed.

BEC Zone and Subspecies Effects on Foliar Secondary Metabolites Populations of *P. c. latifolia* from the Coastal Western Hemlock (CWH) and Interior Cedar Hemlock (ICH) zones had greater levels of soluble phenolics (41% to 45% more) than the Boreal White/Black Spruce (BWBS) zone (F=8.348, P<0.001, N=237) (Fig. 2a). Lignin levels were greater in the BWBS zone (11% to 22% more) than the SBS or Engelmann Spruce/Subalpine Fir (ESSF) zones, and trees from the ICH zone had greater lignin levels (14% more) than those in the ESSF zone (F=4.591, P=0.001, N=238). Total monoterpenoid levels were greater in *P. c. latifolia* from the ICH zone (72% to 242% more) than from any other zone (F=10.105, P<0.001, N=226) (Fig. 2b). Total sesquiterpenoid levels were greater in the CWH zone (60% to 147% more) than any other zone, and levels were greater in the ICH (38% more) and BWBS (37% more) zones than the SBS zone (F=38.467, P<0.001, N=233). Total diterpenoid levels were greater in the CWH population (39% to 857% more) than any other population, and diterpenoid levels were also greater in populations in the BWBS and ICH zones (93% to 588% more) than in the ESSF or SBS zones (F=38.763, P<0.001, N=224).

The foliar levels of almost every individual compound, with the exceptions of sabinene, β-pinene, and linalool, were significantly related to BEC zone (Table 3). Pines from the ICH zone possessed greater levels of camphene, δ-3-carene, and β-phellandrene than pines from the ESSF zone (F>4.088, P<0.010, N=233). Pines in the CWH zone had greater levels of α-cubenene, aristolene, β-cadinene,

Table 2 correlations among the different classes of secondary metabolites analyzed from the foliage of *Pinus contorta latifolia*

	Phenolics *r (P)*	Lignin *r (P)*	Monoterpenoids *r (P)*	Sesquiterpenoids *r (P)*
Lignin, N=237	0.063			
Monoterpenoids, N=224	0.197*	−0.047		
Sesquiterpenoids, N=226–231	0.105	−0.006	0.123	
Diterpenoids, N=217–224	0.124	0.081	0.030	0.800**

* P<0.010; ** P<0.001

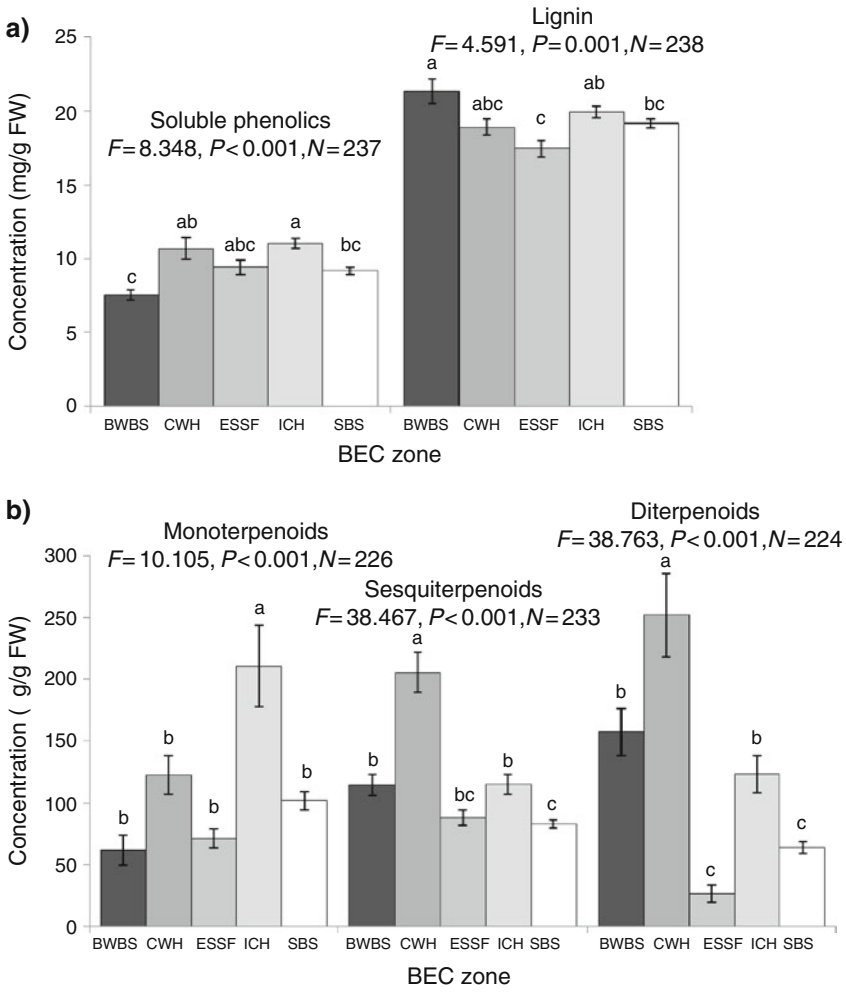


Fig. 2 Concentrations (mean ± s.e.) of **a** phenolic and **b** terpene secondary metabolites in the foliage of *Pinus contorta latifolia* collected from various British Columbia biogeoclimatic (BEC) zones. ANOVA statistics are given, and letters indicate significant differences by Tukey HSD tests. BEC zones are as follows: Boreal White/Black Spruce (BWBS); Coastal Western Hemlock (CWH); Engelmann Spruce/Subalpine Fir (ESSF); Interior Cedar Hemlock (ICH); and Sub-Boreal Spruce (SBS)

and germacrenol than those growing in either the ESSF, ICH, or SBS zones ($F \geq 5.247$, $P<0.001$, $N=233$). Pines growing in the BWBS zone possessed greater levels of the unidentified sesquiterpenoid than those growing in the ESSF or SBS zones ($F=5.355$, $P<0.001$, $N=233$). Pines growing in the CWH zone possessed greater levels of all five diterpenes than pines growing in the ESSF or SBS zones ($F \geq 4.766$, $P<0.001$, $N=233$).

Geographic and Climatic Variables and Secondary Metabolite Levels By using the mean compound levels from each site for the correlation analyses, foliar levels of phenolics were negatively associated with longitude (i.e., phenolic levels were lower in more easterly sites); levels of lignin were positively associated with latitude (i.e., lignin levels were higher in more northerly stands); levels of sesquiterpenoids were positively associated with winter temperature and annual precipitation; and levels of diterpenoids were negatively associated with longitude but positively associated with latitude and winter temperature (Table 4). No other significant correlations were observed.

Considering the foliar levels of SMs in each tree and not using site means for the correlation analyses, levels of phenolics were negatively associated with elevation, longitude, summer temperature, and summer precipitation but positively associated with latitude and winter temperature; levels of lignin were negatively associated with longitude, summer temperature, annual temperature, and annual precipitation but positively associated with latitude; levels of all terpenoid classes were negatively associated with elevation and longitude but positively associated with latitude, summer temperature (with the exception of the diterpenoids), winter temperature, annual temperature, and annual precipitation (with the exception of the monoterpenoids); and levels of monoterpenoids were negatively associated with summer precipitation, whereas levels of sesquiterpenoids and diterpenoids were positively associated with summer precipitation (Table 4). No other significant correlations were observed.

For trees in the SBS zone only, total phenolics were negatively associated with elevation, longitude, summer temperatures, annual temperature, summer precipitation,

Table 3 Mean compound levels from foliage of *Pinus contorta latifolia* (μg/g FW) and ANOVA statistics showing British Columbia's biogeoclimatic zone differences, with letters indicating separation by Tukey HSD tests

Class	Compound	British Columbia Biogeoclimatic Zone					
		Boreal White/ Black Spruce	Coastal Western Hemlock	Engelmann Spruce/ Subalpine Fir	Interior Cedar/ Hemlock	Sub-Boreal Spruce	*F*
Phenolics	total phenolics	7.56±0.36 c	10.71±0.73 ab	9.42±0.49 abc	11.04±0.34 a	9.16±0.23 bc	8.348***
	lignin	21.34±0.86 a	19.00±0.52 abc	17.46±0.58 c	19.92±0.40 ab	19.15±0.27 bc	4.591**
Monoterpenoids	sabinene	0.000±0.000	0.201±0.161	0.008±0.008	0.018±0.018	0.712±0.491	0.339
	α-pinene	2.97±1.03 ab	3.64±0.41 ab	1.14±0.21 b	5.52±1.30 a	4.23±0.43 ab	2.560*
	camphene	2.59±0.54 ab	2.65±0.40 ab	1.18±0.14 b	2.93±0.18 a	2.11±0.16 ab	4.088**
	β-pinene	14.56±4.94	6.11±2.37	11.83±2.72	11.30±4.03	20.93±2.72	2.127
	β-myrcene	1.21±0.49 b	2.38±0.51 ab	1.72±0.22 ab	6.37±1.00 a	3.83±0.66 ab	3.133*
	α-phellandrene	6.43±0.65 a	4.24±0.54 ab	3.68±0.34 b	5.02±0.48 ab	3.97±0.24 b	4.464**
	δ-3-carene	8.01±0.78 ab	6.65±0.52 ab	4.84±0.37 b	9.07±1.13 a	5.31±0.31 b	7.515***
	p-cymene	0.85±0.20 b	2.41±0.43 a	0.98±0.12 b	2.17±0.16 a	1.00±0.10 b	12.994***
	β-phellandrene	20.9±5.2 b	80.1±14.1 b	35.5±5.7 b	156.3±26.6 a	64.9±6.3 b	11.134***
	δ-2-carene	0.17±0.13 b	1.98±0.26 a	0.26±0.12 b	1.40±0.28 a	0.40±0.09 b	13.772***
	linalool	0.85±0.41	2.58±0.72	3.24±0.58	3.39±0.72	3.45±0.44	1.555
	camphor	1.28±0.28 b	2.54±0.32 ab	2.10±0.25 ab	2.62±0.18 a	1.45±0.17 b	5.218***
	bornyl acetate	0.01±0.01 b	1.68±0.45 ab	1.72±0.65 a	1.48±0.36 ab	0.90±0.16 ab	3.194*
	verbanone	1.73±0.38 b	3.85±0.07 a	3.01±0.25 a	3.00±0.18 a	2.00±0.12 b	13.513***
Sesquiterpenoids	unidentified sesquiterpenoid	13.2±3.6 a	11.6±0.8 ab	6.5±0.6 bc	9.2±0.5 abc	7.1±0.5 c	5.355***
	α-cubenene	55.1±5.6 b	110.9±12.7 a	34.9±2.3 bc	50.3±4.7 b	34.9±1.6 c	38.814***
	α-copanene	22.3±1.5 a	23.4±5.0 a	20.2±1.5 ab	20.3±1.8 ab	15.2±0.7 b	5.292***
	aristolene	7.9±1.1 b	27.4±3.3 a	8.9±1.0 b	7.6±0.8 b	6.5±0.4 b	48.630***
	β-cadinene	5.3±1.3 b	13.4±1.3 a	3.3±1.0 b	5.6±0.9 b	7.5±0.7 b	6.091***
	τ-cadinol	8.7±2.6 b	16.1±4.1 ab	11.7±2.0 ab	20.0±1.9 a	10.6±1.0 b	5.247***
	germacrenol	1.46±1.06 ab	4.93±1.44 a	2.35±0.60 b	1.56±0.30 b	1.25±0.25 b	5.416***
Diterpenoids	unidentified diterpenoid A	2.64±0.69 bc	7.91±1.95 a	0.56±0.33 c	4.67±0.80 ab	2.75±0.36 bc	8.037***
	unidentified diterpenoid B	10.91±1.42 a	8.76±1.38 ab	1.38±0.45 d	6.28±0.64 bc	4.94±0.38 c	14.430***
	unidentified diterpenoid C	64.7±13.8 a	40.4±6.5 ab	9.5±2.8 c	20.1±2.6 bc	19.9±2.4 c	12.781***
	unidentified diterpenoid D	29.8±3.0 bc	119.9±30.6 a	9.4±3.1 c	62.9±9.5 b	25.8±2.3 c	18.936***
	unidentified diterpenoid E	49.4±11.4 ab	82.9±10.2 a	5.5±1.8 b	31.4±5.8 b	29.1±6.4 b	4.766**

For lignin and total phenolics, N=238; for all other compounds, N=233.

* P<0.050; ** P<0.010; *** P<0.001.

and annual precipitation but positively associated with latitude; lignin levels were negatively associated with elevation, longitude, summer temperatures, annual temperatures, and summer precipitation but positively associated with latitude; monoterpenoid and sesquiterpenoid levels were positively associated with latitude; and diterpenoid levels were negatively associated with elevation, longitude, summer precipitation, and annual precipitation but positively associated with latitude (Table 4). No other significant correlations were observed.

Linear Regression Modeling to Explain Variance in Foliar Secondary Metabolite Levels Whereas the correlation analyses related individual climatic and geographic variables with the observed levels of the SMs, linear regressions were made to combine multiple factors together to explain the observed levels. Mean foliar phenolic levels at each site were best explained [by having the lowest Bayesian Information Criterion (BIC)] by longitude; lignin levels were best explained by elevation, mean annual temperature, and mean winter temperature; monoterpenoid levels were

Table 4 Pearson's correlations between geographic or climatic variables and mean compound levels per site or individual tree compound levels from an analysis of foliage metabolites from *Pinus contorta latifolia*

Analysis Unit	Compound Class	Elevation	Latitude S→N	Longitude W→E	Summer Temp.	Winter Temp.	Annual Temp.	Summer Prec.	Annual Prec.
Site Means, *N*=12	Phenolics	−0.308	0.249	**−0.706****	−0.337	0.380	−0.218	−0.290	−0.019
	Lignin	−0.120	**0.661***	−0.417	−0.198	−0.150	−0.489	−0.475	−0.306
	Monoterpenoids	−0.483	0.320	−0.478	−0.359	0.274	0.349	0.213	−0.138
	Sesquiterpenoids	−0.552	0.412	−0.501	0.397	**0.687***	0.516	0.258	**0.600***
	Diterpenoids	−0.570	**0.608***	**−0.606***	0.277	**0.581***	0.336	0.702	0.384
Individual Trees	Phenolics, *N*=238	**−0.169****	**0.138***	**−0.395*****	**−0.164***	**0.211****	0.119	**−0.191****	−0.016
	Lignin, *N*=238	−0.059	**0.326*****	**−0.206****	**−0.235*****	−0.074	**−0.151***	−0.098	**−0.151***
	Monoterpenoids, *N*=226	**−0.299*****	**0.191****	**−0.290*****	**0.138***	**0.162***	**0.218****	**−0.220****	−0.082
	Sesquiterpenoids, *N*=233	**−0.414*****	**0.304*****	**−0.374*****	**0.200****	**0.516*****	**0.390*****	**0.307*****	**0.461*****
	Diterpenoids, *N*=224	**−0.441*****	**0.462*****	**−0.465*****	0.100	**0.445*****	**0.261*****	**0.216****	**0.297*****
Individual TreesSBS Zone Only	Phenolics, *N*=138	**−0.394*****	**0.288****	**−0.394*****	**−0.279****	0.008	**−0.279****	**−0.308*****	**−0.308*****
	Lignin, *N*=138	**−0.306*****	**0.335*****	**−0.306*****	**−0.394*****	−0.132	**−0.394*****	**−0.182***	−0.121
	Monoterpenoids, *N*=127	−0.145	**0.207***	−0.145	0.072	0.085	0.051	−0.164	−0.077
	Sesquiterpenoids, *N*=134	−0.069	**0.289****	−0.069	0.143	−0.047	0.033	−0.124	0.042
	Diterpenoids, *N*=127	**−0.372*****	**0.422*****	**−0.372*****	−0.140	−0.036	−0.162	**−0.355*****	**−0.224***

Bold text represents a significant association.

* $P<0.050$; ** $P<0.010$; *** $P<0.001$.

best explained by mean summer precipitation, mean summer temperature, and mean winter temperature; sesquiterpenoid levels were best explained elevation, longitude, mean annual precipitation, mean annual temperature, and mean summer precipitation; and diterpene levels were best explained by elevation, latitude, longitude, mean annual precipitation, and mean summer precipitation (Table 5).

By using the chemical levels found in foliage from individual trees and not using their site means for analyses, foliar soluble phenolic levels were best explained by longitude, elevation, and mean annual temperature; lignin levels were best explained by elevation, mean annual temperature, and mean winter temperature; monoterpenoid levels were best explained by mean summer precipitation, mean summer temperature, and mean winter temperature; sesquiterpenoid levels were best explained by longitude, elevation, mean annual precipitation, mean annual temperature, and mean summer temperature; and diterpenoids were best explained by longitude, latitude, elevation, mean annual precipitation, and mean summer temperature (Table 6).

By using the chemical levels found in foliage from individual trees from the SBS zone only, foliar soluble phenolic levels were best explained by latitude, elevation, and mean summer temperature; lignin levels were best explained by mean annual precipitation, mean annual temperature, and mean winter temperature; monoterpenoid levels were best explained by longitude and mean summer temperature; sesquiterpenoid levels were best explained by elevation and mean summer precipitation; and diterpenoids were best explained by longitude and elevation (Table 7).

It should be noted that in all cases the top three models for each variable had close BIC and AIC values, meaning that one model cannot be considered necessarily better than the others. Additional studies and data collection would be necessary to assess which of the top models would be best for explaining the levels of foliar SMs.

Discussion

Ecosystem type (represented by the BEC zone classifications), geographical location, and climate all significantly impacted at least some of the foliar secondary metabolite levels in the *P. c. latifolia* that were analyzed in this study. Thus, *P. c. latifolia* foliar SMs, which likely contribute to overall fitness, varied with ecosystem, locale, and climate.

BEC zones were associated with differential levels of almost every SM that we analyzed. Trees from the CWH and ICH zones often possessed greater foliar SM levels than trees from other zones, albeit significant differences varied depending on the compound. The CWH and ICH are both defined, in part, as rainforests

Table 5 Top three models as selected by lowest Bayesian Information Criterion (with Akaike's Information Criterion also given) explaining secondary metabolite response variables from an analysis of the foliage of *Pinus contorta latifolia* with the means from each site

Response Variable	Model	R^2	BIC	AIC	Explanatory Variables	β	SE
Phenolics	1	0.498*	42.898	41.425	intercept	15.220***	1.851
					longitude	−5.10E-06*	1.62E-06
	2	0.577*	43.303	41.364	intercept	21.100**	4.871
					latitude	−3.82E-06	2.94E-06
					longitude	−6.86E-06**	2.07E-06
	3	0.572*	43.446	41.506	intercept	15.980***	1.900
					elevation	1.96E-03	1.57E-03
					longitude	−7.07E-06*	2.23E-06
Lignin	1	0.781**	38.604	36.178	intercept	38.121***	4.197
					elevation	−0.004**	0.001
					mean annual temp.	−2.750**	0.540
					mean winter temp.	0.992*	0.327
	2	0.765**	39.453	37.028	intercept	24.110**	4.808
					latitude	5.89E-06*	1.85E-06
					mean annual temp.	−1.654*	0.517
					mean winter temp.	0.749	0.344
	3	0.807*	39.618	36.709	intercept	27.440**	5.846
					latitude	1.22E-05**	3.01E-06
					longitude	5.34E-06	2.60E-06
					mean summer prec.	−1.732*	4.946
					mean winter temp.	0.440	0.283
Monoterpenoids	1	0.538	83.875	81.450	intercept	3.018	36.490
					mean summer prec.	4.571	2.665
					mean summer temp.	−1.179*	0.424
					mean winter temp.	2.341	1.331
	2	0.423	84.043	82.103	intercept	23.087	10.686
					mean annual temp.	−2.750**	2.030
					mean summer temp.	−0.844	0.389
	3	0.515	84.450	82.025	intercept	−24.910	31.730
					longitude	−1.45E-05	9.04E-06
					mean summer prec.	5.506	2.705
					mean summer temp.	−0.717	0.431
Sesquiterpenoids	1	0.889**	59.119	55.724	intercept	−58.900*	21.330
					elevation	1.69E-02*	5.91E-03
					longitude	−3.91E-05**	8.92E-06
					mean annual prec.	0.244**	0.050
					mean annual temp.	−2.750**	1.301
					mean summer prec.	7.019*	2.032
	2	0.909*	59.224	55.345	intercept	−23.560	14.150
					latitude	−7.51E-05*	2.72E-05
					longitude	−8.94E-05*	2.64E-05
					mean annual prec.	0.212*	0.065
					mean annual temp.	−2.750**	5.499
					mean summer prec.	16.570*	5.474
					mean summer temp.	0.858*	0.292
	3	0.925*	59.374	55.009	intercept	−42.290	24.780
					elevation	8.58E-03	9.25E-03
					latitude	−4.52E-05	4.25E-05

Table 5 (continued)

Response Variable	Model	R^2	BIC	AIC	Explanatory Variables	β	SE
					longitude	−7.22E-05	3.26E-05
					mean annual prec.	0.220*	0.066
					mean annual temp.	−2.750**	7.937
					mean summer prec.	13.150	6.661
					mean summer temp.	0.576	0.425
Diterpenoids	1	0.834*	77.258	73.864	intercept	−99.790	44.870
					elevation	0.030	0.013
					latitude	3.87E-05*	1.34E-05
					longitude	−4.07E-05*	1.57E-05
					mean annual prec.	0.297*	0.087
					mean summer prec.	5.399	3.249
	2	0.690**	77.339	75.399	intercept	1.529	12.080
					latitude	2.94E-05*	9.18E-06
					mean winter temp.	2.851*	0.936
	3	0.831*	77.487	74.093	intercept	−89.460	40.600
					elevation	3.10E-02	1.38E-02
					latitude	6.30E-05*	2.13E-05
					longitude	−1.83E-05	9.30E-06
					mean annual prec.	0.230	0.095
					mean annual temp.	−2.750**	3.316

Increasing latitude represents movement from south to north, and increasing longitude represents movement from west to east.
* $P<0.050$; ** $P<0.010$; *** $P<0.001$.

(Meidinger and Pojar, 1991). These ecosystems likely allow *P. c. latifolia* to have a longer growing season and a climate where certain abiotic stresses (e.g., drought) are reduced. Both longer growing season and reduced stress could have resulted in additional resources for the pines to invest in their secondary metabolism (Herms and Mattson, 1992). Furthermore, these ecosystems could have been more favorable to pest development (Allen et al., 1996), which would, due to more frequent pest encounters, impose selective pressure on the lodgepole pines to produce more defense-associated compounds in their foliage (Wallis et al., 2010). This latter hypothesis could be overtly tested by attempting to find associations between foliar pest outbreaks and SMs, potentially with dendrochronological techniques to estimate historical pest outbreak frequency (Welsh et al., 2009). Regardless, it would appear that the need to counter biotic stresses in these ecosystems resulted in selection pressure leading to greater levels of defense-associated SMs, namely monoterpenoids for the Interior Cedar-Hemlock ecosystem and both sesquiterpenoids and diterpenoids for the Cedar-Western Hemlock ecosystem.

Precipitation likely had some influence on the measured levels of SMs because these compounds were extracted from 0.1 g of fresh and not dried material. However, the differences in terpenoid levels from the wettest sites would presumably be even greater than those from drier sites if samples were dried first, as wetter sites (i.e., those from the CWH and ICH) would likely have greater water content in their tissues.

Regardless of ecosystem type, stands in more northerly and westerly locations, and at lower elevations, were associated with higher foliar SM levels. These results do not support the hypothesis that more southerly stands would contain greater levels of SMs (Siska et al., 2002; Andrew and Hughes, 2005; Adams et al., 2009). However, in this study, northern, western, and lower elevation sites included some of the wettest and warmest sites, including the CWH and ICH zones. Indeed, prior studies observed that greater pest activity at lower elevations was associated with plants having increased levels of SMs (e.g., Hobbs and Partridge, 1979; Hengxiao et al., 1999; Heineman et al., 2010), and the CWH and ICH sites were at the lowest elevations of all the sites sampled.

In contrast to the other SMs, levels of lignin were highest in the boreal BWBS ecosystem type, whereas the other metabolites were often at the lowest levels in trees from that zone. Lignin is involved with cell wall thickening and strengthening, which contributes to cold temperature tolerance (Smallwood and Bowles, 2002; Wei et al., 2006). However, lignin was not associated with lower winter temperatures, so other selection pressures might be at play.

Table 6 Top three models as selected by lowest Bayesian Information Criterion (with Akaike's Information Criterion also given) explaining secondary metabolite response variables from an analysis of the foliage of *Pinus contorta latifolia* with data from each individual tree

Response Variable	Model	R^2	BIC	AIC	Explanatory Variables	β	SE
Phenolics	1	0.203***	1117.74	1100.38	intercept	14.170***	1.163
					longitude	−8.12E-06***	1.14E-06
					elevation	0.004***	0.001
					mean annual temp.	0.575**	0.220
	2	0.181***	1118.84	1104.95	intercept	21.200***	2.378
					longitude	−6.92E-06***	1.02E-06
					latitude	−3.85E-06**	1.43E-06
	3	0.180***	1119.09	1105.20	intercept	16.040***	0.927
					longitude	−7.17E-06***	1.09E-06
					elevation	0.002**	0.001
Lignin	1	0.191***	1196.74	1179.38	intercept	38.254***	3.041
					elevation	−0.004***	0.001
					mean annual temp.	−2.767***	0.392
					mean winter temp.	1.002***	0.237
	2	0.169***	1197.66	1183.77	intercept	25.840***	3.511
					latitude	7.24E-06***	1.28E-06
					mean summer temp.	−1.040***	0.248
	3	0.187***	1197.85	1180.49	intercept	24.220***	3.362
					latitude	5.90E-06***	1.29E-06
					mean annual temp.	−1.670***	0.362
					mean winter temp.	0.760**	0.241
Monoterpenoids	1	0.199***	2769.22	2752.11	intercept	37.638	152.368
					mean summer prec.	−1.198***	0.177
					mean summer temp.	45.611***	10.987
					mean winter temp.	23.440***	5.464
	2	0.190***	2771.72	2754.62	intercept	−0.024	0.013
					longitude	−1.44E-04***	3.64E-05
					mean summer prec.	−0.072***	0.017
					mean summer temp.	54.740***	10.940
	3	0.147***	2783.48	2766.38	intercept	−390.100**	137.700
					latitude	9.54E-05	4.96E-05
					mean summer prec.	−0.863***	0.174
					mean summer temp.	49.390***	11.340
Sesquiterpenoids	1	0.502***	2405.02	2380.87	intercept	−591.100***	97.790
					longitude	−3.93E-04***	4.15E-05
					elevation	0.170***	0.027
					mean annual prec.	0.245***	0.023
					mean annual temp.	−34.170***	5.909
					mean summer temp.	70.400***	9.337
	2	0.514***	2405.17	2377.56	intercept	−238.300***	64.690
					longitude	−8.95E-04***	1.22E-04
					latitude	−7.52E-04***	1.25E-04
					mean annual prec.	0.211***	0.029
					mean annual temp.	−167.400***	25.280
					mean summer prec.	0.861***	0.134
					mean summer temp.	165.900***	25.180
	3	0.500***	2406.21	2382.05	intercept	−573.300***	96.810
					longitude	−2.62E-04***	3.44E-05

Table 6 (continued)

Response Variable	Model	R^2	BIC	AIC	Explanatory Variables	β	SE
					latitude	1.62E-04***	2.87E-05
					elevation	0.186***	0.029
					mean annual prec.	0.204***	0.019
					mean summer temp.	39.150***	7.016
Diterpenoids	1	0.481***	2574.87	2550.99	intercept	−1013.000***	177.600
					longitude	−4.09E-04***	6.25E-05
					latitude	3.92E-04***	5.20E-05
					elevation	0.304***	0.052
					mean annual prec.	0.297***	0.034
					mean summer temp.	55.000***	12.910
	2	0.492***	2575.48	2548.18	intercept	−816.800***	185.100
					longitude	−6.84E-04***	7.50E-05
					elevation	0.223***	0.050
					mean annual prec.	0.249***	0.054
					mean annual temp.	−66.360***	11.060
					mean summer prec.	0.594***	0.165
					mean summer temp.	102.200***	17.990
	3	0.480***	2575.60	2551.72	intercept	−905.900***	158.700
					longitude	−1.81E-04***	3.64E-05
					latitude	6.37E-04***	8.32E-05
					elevation	0.310***	0.054
					mean annual prec.	0.228***	0.037
					mean annual temp.	54.430***	13.050

Increasing latitude represents movement from south to north, and increasing longitude represents movement from west to east.
* $P<0.050$; ** $P<0.010$; *** $P<0.001$.

When only the SBS *P. c. latifolia* populations were considered (because most pine plantations are found in that ecosystem type), increases in total soluble phenolics and lignin levels were associated with lower temperatures and rainfall averages, suggesting greater abiotic stress. By contrast, monoterpenoids and sesquiterpenoids were not associated with climatic variables at all, perhaps implying low variance in biological stresses that influence their production in the SBS zone. Increasing latitude was positively associated with the production of all SMs for trees in the SBS zone. Studies are needed to determine the specific factors that might explain this trend, such as increased summer day length, decreased growing season duration, or increases in cold temperature extremes that occur with higher latitude that imparts a selection pressure for greater SM production in pine populations.

Although we observed that geographic and climatic variables did affect foliar SM levels in *P. c. latifolia* populations, the physiological and genetic mechanisms behind these observations remain unclear. Our attempts to form testable regression models proved inconclusive, as the top models did not vary much according to their information criteria (both BIC and AIC values), likely because of the inter-relatedness between all of these variables. This also suggests that these geographic and extrapolated climatic variables are perhaps not the best explanatory variables to use for the levels of foliar SMs.

Future studies should attempt to correlate historic and current abiotic and biotic stress levels of stands of *P. c. latifolia* with their secondary metabolites. Associations with current stress levels would indicate that populations might have responded to local abiotic and biotic stresses by shifting SM production or responding via systemic induced responses (Herms and Mattson, 1992; Koricheva et al., 1998). Alternatively, associations with historical stress levels might reveal that different pine populations may have evolved to produce greater SM levels in response to selection pressures caused by adverse abiotic and biotic stressors (Wu et al., 1996; Hodkinson, 2005; Wallis et al., 2010).

Viewed in light of current efforts to assist the migration of *P. c. latifolia* populations from warmer, southerly areas, to colder, northerly areas (O'Neill et al., 2008), these results are not in and of themselves sufficient to suggest that such efforts are or are not worthwhile. However, these results reveal that variation exists between different populations of

Table 7 Top three models as selected by lowest Bayesian Information Criterion (with Akaike's Information Criterion also given) explaining secondary metabolite response variables from an analysis of the foliage of *Pinus contorta latifolia* with data from each individual trees from the SBS zone only

Response Variables	Model	R2	BIC	AIC	Explanatory Variables	B	SE
Phenolics	1	0.314***	639.21	624.53	intercept	−95.450***	17.490
					latitude	2.867E-05***	4.492E-06
					elevation	0.044***	0.007
					mean summer temp.	3.119***	0.630
	2	0.321***	642.83	625.22	intercept	−85.740***	19.470
					latitude	2.870E-05***	4.487E-06
					elevation	0.041***	0.007
					mean summer temp.	2.804***	0.689
					mean winter temp.	0.380	0.337
	3	0.319***	643.06	625.46	intercept	9.569	4.931
					elevation	0.027***	0.005
					mean annual prec.	0.038***	0.007
					mean summer prec.	−0.094***	0.015
					mean winter temp.	2.327***	0.443
Lignin	1	0.260***	696.44	681.80	intercept	47.823***	5.808
					mean annual prec.	0.008**	0.002
					mean annual temp.	−4.221***	0.659
					mean winter temp.	2.569***	0.596
	2	0.259***	696.74	682.11	intercept	−30.680***	8.470
					latitude	2.288E-05***	4.007E-06
					elevation	0.024***	0.005
					mean summer prec.	0.029***	0.008
	3	0.253***	697.91	683.28	intercept	30.851***	6.086
					mean annual temp.	−7.558***	1.744
					mean summer temp.	2.843**	1.011
					mean winter temp.	3.358***	0.822
Monoterpenoids	1	0.152***	1480.66	1469.29	intercept	−225.200*	109.800
					longitude	−3.899E-04***	8.416E-05
					mean summer temp.	58.710***	13.420
	2	0.173***	1482.29	1468.07	intercept	−479.000**	179.600
					longitude	−3.335E-04***	8.927E-05
					latitude	1.931E-04	1.087E-04
					mean summer temp.	58.360***	13.310
	3	0.173***	1482.30	1468.08	intercept	−218.400*	108.900
					longitude	−5.354E-0***4	1.171E-04
					mean annual prec.	0.173	0.098
					mean summer temp.	62.690***	13.490
Sesquiterpenoids	1	0.207***	1334.92	1323.33	intercept	410.870***	56.091
					elevation	−0.284***	0.050
					mean summer prec.	−0.384***	0.079
	2	0.236***	1334.96	1320.48	intercept	−333.200***	74.930
					latitude	1.964E-04***	4.335E-05
					mean summer prec.	−0.253**	0.083
					mean summer temp.	21.750***	4.281
	3	0.234***	1335.22	1320.73	intercept	−450.100***	86.250
					latitude	2.720E-04***	4.571E-05
					mean annual prec.	−0.094**	0.031
					mean summer temp.	24.160***	4.842

Table 7 (continued)

Response Variables	Model	R2	BIC	AIC	Explanatory Variables	B	SE
Diterpenoids	1	0.296***	1344.34	1332.96	intercept	681.500***	93.760
					longitude	−2.618E-04***	3.627E-05
					elevation	−0.397***	0.075
	2	0.308***	1347.07	1332.85	intercept	−459.200***	90.570
					latitude	5.178E-04***	7.736E-05
					mean annual prec.	−0.147***	0.035
					mean annual temp.	35.980***	8.710
	3	0.307***	1347.33	1333.11	intercept	493.500**	168.000
					longitude	−3.047E-04***	4.820E-05
					elevation	−0.320***	0.094
					mean summer temp.	13.340	9.906

Increasing latitude represents movement from south to north, and increasing longitude represents movement from west to east.
* $P<0.050$; ** $P<0.010$; *** $P<0.001$.

P. c. latifolia, especially those in different broadly-defined ecosystem types. Previous studies have shown that when grown in the same location, pines originating from different stands and ecosystems still retain their variance in both SM levels and pest resistance (Wu et al., 1996, 2005; Wallis et al., 2010). Therefore, the variance in SM concentrations observed in pine populations in this study could be predicted to remain for at least one generation if trees undergo assisted migration.

Climate change is predicted to increase both biotic and abiotic stresses on pine populations. Bark beetle populations are the major cause of area wide mortality for *P. c. latifolia*, and these populations are predicted to increase regionally with climate change (Bentz et al., 2010). Coupled with changes in temperature and rainfall patterns, bark beetles could force pine ecosystems to higher latitudes and elevations (Bentz et al., 2010). In this study, terpenoid levels, and to some extent the soluble phenolics and lignin, were greater in ecosystems that were wetter and warmer (the CWH and ICH *versus* the SBS and ESSF), i.e., those likely facing greater biological stresses (Allen et al., 1996). Therefore, migrant pines from these populations would be expected to have a fitness advantage over native pines (i.e., trees whose ancestors originate from the same area) at higher latitude or elevation sites when predicted bark beetle, and possibly pathogen, outbreaks expand into those areas (Bentz et al., 2010). Hypothetically, migrant pines would possess a greater capacity to produce defense-associated compounds. These pines might also be more tolerant of certain abiotic stresses, such as increased temperature extremes and more frequent droughts. However, only longer-term monitoring of the variation of secondary metabolites in multiple stands can confirm if this is the case, or if the native pines might fare little better than migrant trees.

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Host Sex Discrimination by an Egg Parasitoid on Brassica Leaves

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Abstract Egg parasitoids are able to find their hosts by exploiting their chemical footprints as host location cues. In nature, the apolar epicuticular wax layer of plants that consists of several classes of hydrocarbons serves as the substrate that retains these contact kairomones. However, experiments on chemical footprints generally have used filter paper as substrate to study insect behavior. Here, we explored the ability of *Trissolcus basalis* (Scelionidae) females to discriminate between footprint cues left by male and female *Nezara viridula* (Pentatomidae) on leaves of their host plant *Brassica oleracea* (broccoli). Furthermore, we analyzed the chemical composition of the outermost wax layer of broccoli leaves to evaluate the degree of overlap in insect and plant cuticular hydrocarbons that could lead to masking effects in the detection of footprint cues. Our results showed that *B. oleracea* epicuticular wax retains the chemical footprints of adult bugs and allows *T. basalis* females to differentiate hosts of different sex. Traces of female bugs elicited more extensive searching behavior in egg parasitoids than traces of males. The application of *n*-nonadecane, a compound specific to male *N. viridula*, on the tarsi of female bugs prevented parasitoid females from distinguishing between host male and host female footprints. Analyses of *B. oleracea* leaves revealed that epicuticular waxes were mainly composed of linear alkanes, ketones, and secondary alcohols. Alkanes were dominated by *n*-nonacosane (*n*C29) and *n*-hentriacontane (*n*C31), while male-specific *n*-nonadecane (*n*C19) was absent. The ecological significance of these results for parasitoid host location behavior is discussed.

Key Words *Brassica oleracea* · *Nezara viridula* · *Trissolcus basalis* · Host sex discrimination · *n*-nonadecane · Epicuticular waxes · Cryo-method

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Introduction

Insect parasitoids that attack and develop within non-feeding host stages, such as eggs or pupae, have evolved the ability to exploit chemical cues related to the adult stage. This behavior can help to overcome the so-called reliability-detectability dilemma (infochemical detour) (Vet and Dicke, 1992). In the case of hymenopteran egg parasitoids, an extensive body of literature describes how parasitoids can find patches where eggs have recently been deposited or where this is soon to occur, chiefly by exploring the semiochemicals produced by host adults (reviewed by Fatouros et al., 2008; Colazza et al., 2010). Within the host's habitat, volatile pheromones may be detected by female egg parasitoids to actively reach the mating sites of hosts (Lewis et al., 1982; Colazza et al., 1997; Boo and Yang, 2000; Conti et al., 2010) or to recognize mated female hosts ready to lay eggs. Such gravid females also may be used for phoretic transportation (Fatouros et al., 2008; Huigens et al., 2010). Contact kairomones left by mated female hosts allow egg parasitoids to locate recently laid eggs more efficiently (Colazza et al., 1999; Chabi-Olaye et al., 2001; Fatouros et al.,

2007). Furthermore, plants can modify the synthesis of organic defensive compounds as a consequence of the insect footprints (Bown et al., 2002).

From a tri-trophic perspective, chemical substances deposited by many insects while walking on a plant surface can mediate both intra- and interspecific relationships (Klomp, 1981; Colazza et al., 1999; Witjes and Eltz, 2009). The physicochemical characteristics of plant surfaces should, therefore, strongly influence these interactions, as the chemistry of the epicuticular wax components may affect how exogenous substances are adsorbed and released (Müller and Riederer, 2005). For example, the larval parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) responds differently to the chemical footprints of its host *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), when footprints that mainly consist of linear and monomethyl-branched alkanes are left on the leaf surface of either wild-type or *eceriferum* mutants of barley. The mutants were characterized by lower amounts of the main wax component hexacosanol and a high aldehyde fraction (Rostás et al., 2008; Rostás and Wölfling, 2009).

In recent years, the role of footprint cues deposited by true bugs (Hemiptera: Heteroptera) as an indirect host-related contact cue for scelionid egg parasitoids (Hymenoptera: Scelionidae) has been investigated (reviewed by Colazza et al., 2010). Extensive studies have been carried out in particular on the host-parasitoid association of *Nezara viridula* (L.) (Heteroptera: Pentatomidae) and *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae). In these experiments, the behavioral responses of the parasitoid were observed in arenas made of filter paper, which is an amorphous artificial substrate that does not interfere with parasitoid behaviors and/or the chemical properties of host cues (Colazza et al., 1999; Peri et al., 2006; Salerno et al., 2006). Under such conditions, *T. basalis* females show an initial motionless period of a few minutes (arrestment response) followed by locomotion and increased antennal movements while searching within the patch (motivated search). During the motivated search, wasp females adjust both turning rate (klinokinesis) and locomotion rate (orthokinesis) depending on the host's sex. The presence of contact kairomones eventually leads to a longer and more intensive exploration in an area contaminated by the host adult females, as the cues associated with this host sex are spatially and temporally closer to the host eggs (Colazza et al., 1999).

Under natural conditions, the interactions between *N. viridula* and *T. basalis* are influenced by the presence of plant surface waxes. Previous experiments demonstrated that the wasps responded to footprints from female *N. viridula* walking on *Vicia faba* L. plants only if the leaf's wax layer was intact, but were unable to detect host kairomones when the bugs have walked over mechanically dewaxed leaves (Colazza et al., 2009). Clearly, the physicochemical characteristics of the leaf surface can hamper the detectability of host footprints by wasp females, as active compounds could be part of the components that make up the plant's epicuticular wax surface. Recent observations of *T. basalis* behavior using filter paper arenas demonstrated that the linear alkane *n*-nonadecane (nC_{19}) is an active component among the cuticular hydrocarbons of *N. viridula* males that allows wasp females to distinguish between residues left by male or female bugs (Colazza et al., 2007). As linear alkanes are a major class of compounds in plant epicuticular waxes (Eigenbrode and Espelie, 1995; Müller and Riederer, 2005)—although alkanes with less than 21 carbon atoms don't seem to be widely present—possible masking effects by the alkanes present in the plant waxy background on *N. viridula* male-specific hydrocarbons need to be assessed.

In this study, we investigated the ability of *T. basalis* females to distinguish between the footprint cues left by male and female *N. viridula* on leaves of broccoli plants (*Brassica oleracea* L., var. Italica, cv. Marathon). Then, we evaluated the responses of female wasps to the male bug-specific hydrocarbon *n*-nonadecane. Finally, we studied the chemical composition of the broccoli leaf surface to evaluate its role in mediating the interactions between host footprints and female wasps and specifically to answer the question whether *n*-nonadecane was present or absent.

Methods and Materials

Plants In all experiments, broccoli plants grown from certified seed material (Esasem Spa, Casaleone -VR- Italy) were used. In experiment 1, plants were cultivated in 14-cm plastic pots with fertilized commercial potting soil (Trflor, HOCHMOOR) in a climate-controlled greenhouse of the Department of DEMETRA, University of Palermo, Italy from 08/2008 to 12/2008. In experiments 2 and 3, plants were grown in 9-cm-pots containing soil mixture ED 73, and kept in a growth chamber with a day/night cycle of 16:8 hr, 20°C temperature, and 60% relative humidity at the Department of Botany II, University of Würzburg, Germany.

Insects Colonies of *N. viridula* and *T. basalis* were established from material collected in the fields around Palermo, Italy, and were maintained in separate climate chambers at 26±1°C, approximately 60% R.H., and a photoperiod of 16:8 hr (L:D). Immatures and adults of *N. viridula* were reared in wooden cages (50×30×35 cm with 5-cm circular mesh-covered holes) and fed with seasonal fresh vegetables, and sunflower seeds. Mated adults of known age were available for the bioassays by keeping pairs of *N. viridula* in different cages, and checking daily until mating was assured. Females and males then were

separated and placed into different rearing cages. Egg masses of *N. viridula* were collected from the rearing cages on a daily basis and used to maintain both *N. viridula* and *T. basalis* colonies. Newly laid egg masses of *N. viridula* were individually exposed to 3–4 female wasps for about 3 d in 16-ml glass tubes. Then, parasitoids were removed and parasitized egg masses were held in the rearing room until adults emerged. Adults of *T. basalis* were fed with a 10% honey-water solution streaked on the inside surface of the glass tubes.

Experiment 1 The aim of this experiment was to determine whether female parasitoids were able to detect and discriminate the chemical footprints of male and female bugs on the surface of broccoli leaves. Four-wk-old plants with 5–6 fully expanded leaves were exposed to 2–3 *N. viridula* adults. The stink bugs were allowed to walk over the leaves and produce chemical footprints for about 30 min according to the method of Colazza et al. (2009). Plants not exposed to insects were used as controls. Male and female bugs were 8–10 d-old kept isolated for 3–4 d after mating. *Trissolcus basalis* females were 4 d-old, mated and naive with respect to both, oviposition experience and contact with host chemical traces. The day before the experiments were carried out, single wasps were isolated in 2-ml glass vials closed with a cotton plug. Parasitoids were fed with a drop of honey provided on the inside surface of the vials. Thirty to sixty min prior to the experiments, female wasps were transferred to the bioassay room to acclimatize. A single wasp was released in the center of an adaxial leaf surface by gently tapping on the vial. Wasps were scored either as "responding" if they immediately displayed the typical arrestment posture, i.e., remaining motionless with the antennae in contact with the leaf surface, or as "non-responding" where they did not show this posture after three trials. For responding females the time spent on the adaxial leaf surface was measured as "retention time" with a stopwatch. Each leaf was used for testing five wasp females. Thirty replicates were carried out for each treatment. All experiments were conducted at room temperature (20–25°C) and under natural daylight conditions between 9:00 a.m. and 2:00 p.m.

Experiment 2 In a series of bioassays we evaluated the effect of *n*-nonadecane (nC_{19}), the compound specific for male bugs, on the discriminatory behavior of female parasitoids. Females of *N. viridula* were immobilized by cooling the insects down at −20°C for 5 min. Then, three tarsi on one side of the insect body were treated with hexane (nC_6) or with 4 µl of the test alkanes (0.1% w/v in hexane), while the remaining three tarsi were left untreated. Tested alkanes were: *n*-nonadecane (*n*C19) (Fluka, 99.8% purity) to mimic footprints of male bugs, and *n*-eicosane (nC_{20}) (Sigma-S. Louis, MO, USA, 99% purity) a saturated hydrocarbon that is not present in adult *N. viridula* cuticle but was used as a control for any masking or modifying effects on host footprint detection by *T. basalis.* The pure solvent was applied as a second control. Female bugs retained normal activity 10–15 min after treatment, and then they were left to walk over the adaxial surface of a leaf disk (5 cm diam) cut out from a fully expanded broccoli leaf for 30 min. Finally, a female parasitoid was released in the center of the leaf disk and its behavior was scored as described in the previous experiment. Each leaf disk was used for testing five wasps. Thirty replicates were carried out for each treatment. All experiments were conducted between 9:00 a.m. and 2:00 p.m. at room temperature (20–25°C) and under natural daylight conditions.

Experiment 3 The compounds that constitute the epicuticular wax layer of broccoli leaves were assessed, in particular, to answer the question whether *n*-nonadecane was present. Leaf surfaces were prepared by mechanically removing the outermost wax layer, using the cryo-adhesive based method described by Riedel et al. (2003). Disks were cut out from broccoli leaves taken from 4-wk-old plants with 5–6 fully expanded leaves. Leaf disks were placed on an aluminum holder. Then, cylinders (inner diam: 1.4 cm) were centered on the leaf disks and filled with water, completely covering the surface of the disks. The whole apparatus was frozen in liquid nitrogen and the resulting ice blocks were taken off the leaf disks by lifting the cylinders, thereby stripping off the wax. The cylinder walls were rapidly warmed up thus allowing the ice blocks to slip out. The wax-holding ice blocks were immediately collected in a vial containing chloroform (4 ml) and water (4 ml). Preparations from 3 to 5 leaf disks from the same leaf were pooled and *n*-tetracontane (nC_{40}) was added as an internal standard. The two-phase system was heated to approximately 40°C in a water bath and agitated repeatedly. Then, the organic phase was removed and the aqueous phase was extracted × 3 with chloroform. All three chloroform fractions were combined. The wax extracts in chloroform were dried over Na_2SO_4 to eliminate all remaining water and were then filtered. Prior to gas chromatographic (GC) analysis, chloroform was evaporated from all samples under a gentle stream of N_2 while heating the sample-vials to 50°C. Then, all samples were treated with bis-*N,N*-(trimethylsilyl)-trifluoroacetamide in pyridine (BSTFA, Supelco, Sigma-Aldrich) (30 min at 70°C) to transform all hydroxyl-containing compounds to their corresponding trimethylsilyl (TMSi) derivatives. The qualitative composition was analyzed using a capillary GC (6890 N, Agilent Technologies, Santa Clara, CA, USA; column: 30 m×0.32 mm inner diam, Zebron ZB-1HT, d_f=0.1 µm, Phenomenex, Torrance, CA, USA) and mass spectrometric

detector (MSD 5973 N, Agilent Technologies, Santa Clara, CA, USA; 70 eV, m/z 50–650). Gas chromatography was carried out with cool-on-column injection at 50°C. Oven temperature was held at 50°C for 2 min, raised by 3°C min^{-1} to 320°C and held for 20 min at 320°C. Helium carrier gas inlet pressure was 50 kPa at injection, held for 5 min, raised by 3 kPa min^{-1} to 150 kPa and held at 150 kPa for 90 min. Wax components were identified by comparison of their mass spectra with those of authenticated standards and literature data. Quantification of wax compounds was accomplished by capillary GC (6850 N, Agilent Technologies, Santa Clara, CA, USA; same column as above) and flame ionization detection under the same gas chromatographic conditions as described above but with H_2 as carrier gas. The wax composition of six individual plants was analyzed.

Statistical Analysis The total numbers of "responding" and "non-responding" *T. basalis* females for each treatment were compared with Pearson's χ^2 test. Goodman's *post hoc* procedure was used for internal contrasts among different treatments. The wasp's resident time on leaves and leaf disks, recorded only from responding individuals, led to unequal numbers of replicates. Therefore, these unbalanced data were analyzed using one-way ANOVA with type III sums of squares for log-transformed data. Comparisons among means were made using Fisher's LSD *post hoc* tests. Statistical analyses were performed using XLSTAT add-in software for Excel.

Results

Experiment 1 Wasp responses to broccoli leaves were strongly affected by the presence or absence of host chemical footprints ($\chi_{(2)}^2$=32.76; P<0.001; Pearson's χ^2) (Fig. 1 a). On leaves contaminated with *N. viridula* footprints, about 75% of *T. basalis* females displayed the typical arrestment posture, while on non-contaminated leaves only ca. 13% responded. No statistical differences could be observed when comparing the responses of wasps towards male or female hosts. In contrast, the average leaf residence time of responding wasps was clearly influenced by the host's sex ($F_{(2,47)}$=6.38, P=0.002; ANOVA Type III Sum of Squares analysis) (Fig. 1 b). On average, responding wasps showed longer retention time on leaves when footprints originated from female rather than male *N. viridula*. No statistical difference in retention time was found between uncontaminated leaves and leaves contaminated with male footprints.

Experiment 2 About 80% of all parasitoids displayed the typical arrestment posture on encountering leaf disks treated with chemical footprints from *N. viridula* females. This response was irrespective of whether bug legs had been treated with *n*-nonadecane, *n*-eicosane, or hexane ($\chi_{(2)}^2$=0.53, P=0.76; Pearson's χ^2) (Fig. 2 a). In contrast, tarsal treatments significantly affected the retention time of the wasps exploring the treated leaf disks. Host footprints from females that had been treated with *n*-nonadecane induced parasitoids to search for a significantly shorter period than the footprints from bugs treated with *n*-eicosane or hexane ($F_{(2,68)}$=5.62, P<0.001; ANOVA Type III Sum of Squares analysis) (Fig. 2 b). The retention time of the latter two treatments was similar with no significant difference between them.

Experiment 3 The total amount of epicuticular waxes on the adaxial side of *Brassica* leaves averaged 11.60 μg cm^{-2}±

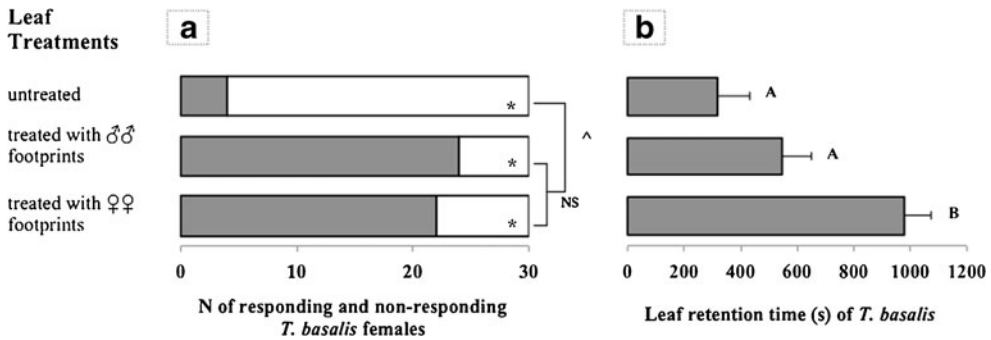


Fig. 1 Effects on the host sex discrimination ability of *Trissolcus basalis* females exploring the adaxial leaf surface of broccoli. Leaves were contaminated with chemical footprints laid by adult males and females of *Nezara viridula*. The number of tested wasp females was 30 for each treatment. **a** Bars indicate the number of the wasps that showed the arrestment response (responding, *grey bars*) and those that did not show this behavior (non-responding, *white bars*). Statistically significant differences are indicated with asterisks within treatments, and by carets between treatments. NS = not significant. Karl Pearson χ^2 test and Goodman's *post hoc* procedure. **b** Bars indicate mean values, whiskers are ± SE, of the leaf resident time of *T. basalis* females that responded to the different treatments. Bars followed by the same letters are not significantly different. One-way analysis of variance with type III sums of squares, followed by Fisher's LSD *post hoc* tests

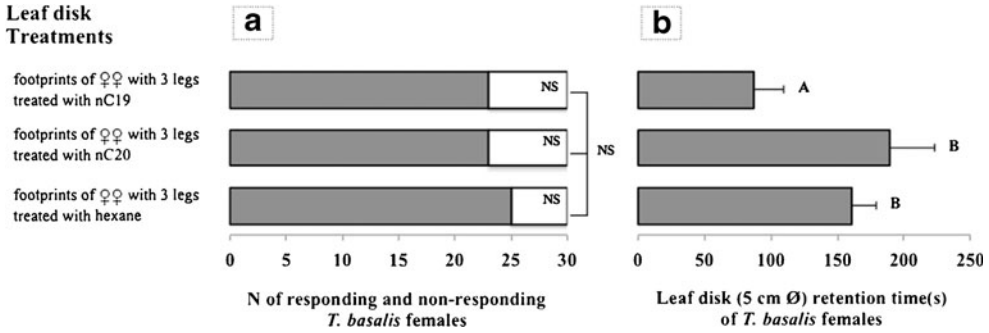


Fig. 2 Effects on the host sex discrimination ability of *Trissolcus basalis* females exploring leaf disks (5 cm Ø) of broccoli. Leaves were contaminated with chemical footprints of *N. viridula* females with legs treated with *n*-nonadecane, *n*-eicosane, and hexane, respectively. The number of tested wasp females was 30 for each treatment. **a** Bars indicate the number of wasps that showed the arrestment response (responding, *grey bars*) and those that did not show this behavior (non-responding, *white bars*). Statistically significant differences are indicated by asterisks within treatments, and by carets between treatments. NS = not significant. Pearson's χ^2 test and Goodman's *post hoc* procedure. **b** Bars indicate mean values, whiskers are ± SE, of the leaf resident time of *T. basalis* females that responded to the different treatments. Bars followed by the same letters are not significantly different. One-way analysis of variance with type III sums of squares, followed by Fisher's LSD *post hoc* tests

1.50 (mean ± SE). The mixture was composed mainly of linear hydrocarbons (38.70%), ketones (28.11%), and secondary alcohols (12.06%). Small amounts of esters (4.86%), primary alcohols (1.75%), aldehydes (1.56%), and traces of fatty acid esters and saturated and unsaturated fatty acids (less than 1%) were found. Within the class of linear hydrocarbons, *n*-nonacosane (nC_{29}) was the most abundant component, followed by *n*-hentriacontane (nC_{31}), while *n*-nonadecane was not present at the detection level in the extracts (Fig. 3).

Discussion

Here, we provide further evidence that broccoli epicuticular wax retains chemical footprints of adult *N. viridula* and show that these cues allow *T. basalis* females to distinguish between the sexes of their host. On encountering the chemical footprints of adult bugs, motivated egg parasitoids displayed a well-described arrestment response that was followed by extensive host searching behavior. More time was spent investigating leaves contaminated by female than

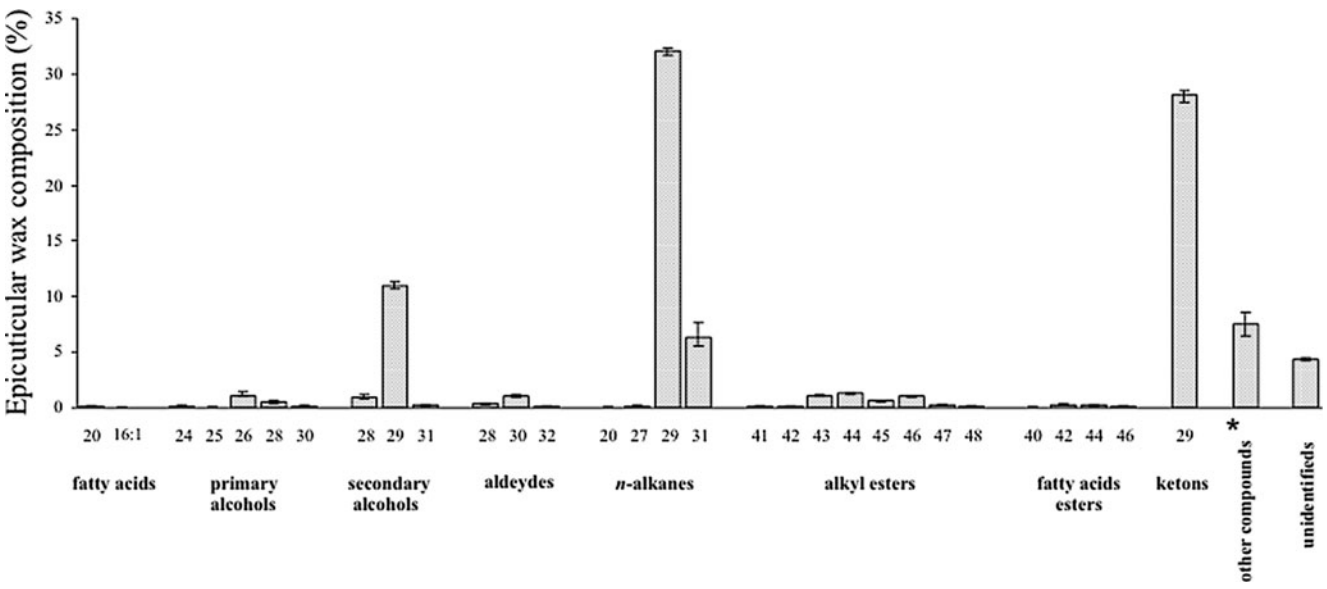


Fig. 3 Composition (%) of the outer epicuticular wax layer on the adaxial leaf surface of *Brassica oleracea* var. Italica cv. Marathon. Relative amounts of compounds are given as medians (*bars*) and 25th and 75th percentile (*whiskers*) (N=6). Compounds are listed according to chain lengths in the homologous series of aliphatic compound classes. *Compounds not separable in GC peaks

by male hosts or uncontaminated control leaves. By using an approach based on tarsal treatments of adult bugs, our results support previous experiments conducted on filter papers, which indicated that *n*-nonadecane (nC_{19}), a compound present only in *N. viridula* males, plays a role as a host sex recognition cue for wasp females. This conclusion can be drawn because treating the legs of female *N. viridula* with *n*-nonadecane resulted in footprints that apparently resembled male footprints. Female parasitoids were unabale to differentiate between male footprints and female footprints mimicking those of males. It remains to be investigated for how long *T. basalis* can detect the chemical footprints of its host. Studies on a larval parasitoid showed that the wasps recognize footprints from caterpillars for a period of at least 2 days (Rostás and Wölfling, 2009). This suggests that these kairomones can be retained for a relevant length of time and should help to improve the efficiency in finding a suitable host.

So far, the linear alkane *n*-nonadecane has been found in few pentatomid species (Aldrich, 1996; Durak and Kalender, 2007; Durak, 2008). In *N. viridula*, it is present only in adult males. It occurs in the sex pheromone blend where it represents about 7% of the total volatiles (Aldrich et al., 1987), and it constitutes about 3% of the amount of cuticle linear hydrocarbons (Colazza et al., 2007). Long-chain hydrocarbons are of high volatility, thus *n*-nonadecane from *N. viridula* can disperse into the environment as a component of the pheromone blend and adsorb onto plant surfaces at the time the adults touch the plants. Earlier experiments showed that both male volatile pheromone and male contact footprints stimulate wasp females. However, when compared, *T. basalis* clearly preferred the chemical footprints produced by host females (Colazza et al., 1999). Therefore, the presence of this linear alkane in different sources, i.e., in the pheromone glands and the cuticle of the male bug, and the similar responses of *T. basalis* females to pheromone and footprints, suggests a function for *n*-nonadecane as a compound to differentiate between host sexes. Linear hydrocarbons most likely are secreted from cuticle tissue to protect the insect from desiccation (Howard and Blomquist, 2005). However, whether *n*-nonadecane is passively dispersed as a component of the cuticular hydrocarbons or if it is released through active secretion from the pheromone glands, is as yet unknown.

All compound classes and homologues identified from the wax extracts of broccoli leaves are found commonly in plant waxes (Müller and Riederer, 2005). The wax mixture contained mainly linear hydrocarbons, ketones, and secondary alcohols. Linear hydrocarbons were dominated by *n*-nonacosane and *n*-hentriacontane, while *n*-nonadecane was absent. Based on the literature, *n*-nonadecane is not present in the epicuticular waxes of several of *N. viridula* host plants belonging to different families, such as Solanaceae, e.g., *Lycopersicum esculentum* (Zygadlo et al., 1994), *Solanum tuberosum* (Szafranek and Synak, 2006); Brassicaceae, e.g., *B. oleracea* (Baker, 1974; Hunsche et al., 2006; Purdy and Truter, 2010); Fabaceae, e.g., *Vicia faba* (Powell et al., 1999), *Glycine max* (Kim et al., 2007), *Phaseolus vulgaris* (Hunsche et al., 2006), and *Pisum sativum* (Gniwotta et al., 2005). Therefore, the absence of *n*-nonadecane among the hydrocarbons of epicuticular plant surfaces of these *N. viridula* host plants avoids possible masking effects and may enhance *T. basalis* host location.

Our study represents a starting point in elucidating the mechanisms by which egg parasitoids can distinguish host footprint compounds from the waxy background of the plant cuticle, but further studies are necessary to clarify the role that plant cuticular hydrocarbons play in the host finding process of parasitoids. Recent experiments indicate that footprints of *N. viridula* females also contain diglycerides and triglycerides of high molecular weight, and long-chain alcohols and fatty acids (Lo Giudice, 2009). On the various host plant species, these compounds can be retained to different degrees as a result of the absorptive properties of epicuticular waxes. As a consequence, the host recognition behavior of *T. basalis* females may vary among plants. Another interesting question that awaits better clarification is, whether *T. basalis* is not only able to recognize the adult host's sex but also the physiological state of the female or distinguish adults from immatures. Such capabilities were demonstrated in the related species *Trissolcus brochymenae* (Hymenoptera: *Scelionidae*) and its host *Murgantia histrionica* (Heteroptera: Pentatomidae). Salerno et al. (2009) found that footprints of mated female bugs elicited longer periods of host searching than kairomones from virgin bugs. However, as in most experiments, the chemical footprints were tested on filter paper substrate, while future studies on parasitoid-host interactions should take into account the great variety of plant-specific epicuticular wax chemistries and how they may potentially influence the perception of kairomones.

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Behavioral and Chemical Investigations of Contact Kairomones Released by the Mud Dauber Wasp *Trypoxylon politum*, a Host of the Parasitoid *Melittobia digitata*

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Abstract Contact kairomones from the host mud dauber wasp *Trypoxylon politum* Say (Hymenoptera: Crabronidae) that mediate behavioral responses of its ectoparasitoid *Melittobia digitata* Dahms (Hymenoptera: Eulophidae) were investigated. Chemical residues from host by-products, the cocoon, and the meconium, induced arrestment behavior of macropterous female parasitoids, while those from the host stage attacked, i.e., the prepupa, did not. *Melittobia digitata* response to polar and apolar extracts of host by-products indicated kairomone(s) solubility mainly in hexane. GC and GC/MS analysis of cocoon and meconium apolar extracts revealed a mixture of linear carboxylic acids from C_6 to C_{18}, and both extracts contained almost identical compounds. When a reconstructed blend of host by-product carboxylic acids was tested, *M. digitata* females showed only a weak response, thus suggesting that other unidentified compounds present in small quantities also may be involved. *Melittobia digitata*'s response to contact kairomones was innate and not affected by previous host exposure experience. Our results provide evidence of contact kairomone exploitation in the genus *Melittobia*. The ecological significance of these findings in the host selection process of *M. digitata* is discussed.

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Introduction

Insect parasitoids rely mainly on semiochemicals for location and recognition of hosts, and several studies have been published on the role played by various chemical cues in the host selection behavior of hymenopteran parasitoids (Godfray, 1994; Quicke, 1997; Vinson, 1998, 2010; Fatouros et al., 2008). Parasitoids, at the initial steps of the host location process, exploit mainly volatile cues (Group I cues sensu Vinson, 1998, 2010) since they can be perceived at long range distance, whereas slightly volatile cues or contact cues become progressively more important when parasitoids are in the close vicinity (Group II cues) or in contact (Group III cues) with the host (Vinson, 1998, 2010). Experience is particularly important in affecting the initial phases of host selection behavior, especially for generalist species, as experienced females have been reported to respond more efficiently to the host-plant complex than naive females (Drost et al., 1986; McAuslane et al., 1991). However, the role of experience may be important at all levels of the host selection process (i.e., time required to recognize and parasitize a host decreases as the parasitoid handles successive hosts) (Wajnberg, 1989; Segoli et al., 2009).

Wasps in the genus *Melittobia* are gregariously developing ectoparasitoids that frequently attack solitary wasps and bees, but also attack a wide range of hosts that represent several insect orders (González et al., 2004; Matthews et al., 2009). *Melittobia* wasps are sexually

dimorphic, and females are polymorphic (short winged and long winged) (González and Matthews, 2005, 2008; Matthews et al., 2009). Long-winged females, also called macropterous females, are the dispersal stage in all *Melittobia* species, while some non-dispersing short-winged females, also called brachypterous females, may be produced on high quality hosts. While several aspects of *Melittobia*'s behavioral ecology have been studied (reviewed in Matthews et al., 2009; Tanner et al., 2011), the chemical ecology of *Melittobia* species is poorly understood. The only such study (Cônsoli et al., 2002) elucidated the role of α- and β-*trans*-bergamotenes as the male-produced sex pheromone in *M. digitata* Dahms. Nonetheless, chemical cues are likely to be important in allelochemical communication of *Melittobia* too, as, for example, during the host location process. The antennae of *Melittobia* females possess numerous mechano- and chemo-receptors (Dahms, 1984; Imandeh et al., 2001), suggesting that chemical cues are important in *Melittobia* biology.

Macropterous females of *Melittobia* wasps appear to disperse randomly once out of their natal nest, but when close to host nests, little is known about cues that might direct them to parasitize a healthy host (González and Terán, 2001; Matthews et al., 2009). However, some progress has been made in understanding how *Melittobia* wasps locate their hosts (Silva-Torres et al., 2005a,b; Cusumano et al., 2010). Cusumano et al. (2010), working with *M. digitata*, suggest that once the mud dauber wasp host *Trypoxylon politum* Say (Hymenoptera: Crabronidae), is located the *Melittobia* female adopts a "sit and wait" strategy relying mainly on indirect host-related volatile cues. Females search for *T. politum* nests under construction, and once located, they enter the newly built cell and wait inside until the host becomes a prepupa, the stage attacked by the parasitoid. Before such transformation, the mature larva scrapes grains from the cell wall and glues them with saliva and the first released meconial fluids to build its cocoon, and once it is built, the larva releases the remainder of the meconium (Cooper, 1957; Cross et al., 1975; R.W. Matthews pers. comm.). The presence of the cocoon and the final release of meconium are indicators associated with host suitability (Cusumano et al., 2010).

Contact cues are important in host location, host recognition, and also in mate finding of parasitic wasps (Godfray, 1994; Fauvergue et al., 1995; Vinson, 1998; Colazza et al., 1999). The parasitoid response to contact cues usually consists of flight inhibition, an intense tapping of the substrate with its antennae, and a modification of the walking pattern characterized by a reduction in linear speed and an increase in turning rate (arrestment response) (Waage, 1978, 1979). Several host-produced stimuli act as contact cues, such as frass (Loke and Ashley, 1984; Takabayashi et al., 1985; Meiners et al., 1997), honeydew (Vinson et al., 1978; Gardner and Dixon, 1985; Mehrnejad and Copland, 2006), wing scales (Laing, 1937; Noldus and van Lenteren, 1985; Gardner and van Lenteren, 1986; Schmidt and Carter, 1992; Chabi-Olaye et al., 2001), footprints (Colazza et al., 1999; Borges et al., 2003; Conti et al., 2003; Rostás and Wölfling, 2009), and mandibular gland secretions (Waage, 1978).

Chemical investigations have revealed that both polar and non-polar compounds can be the biologically active substances responsible for eliciting wasps' arrestment behavior. For example, active compounds in honeydew are often water or polar solvent soluble (Vinson et al., 1978; Bouchard and Cloutier, 1984); water-soluble propriety can be important for parasitoids because of rain that can wash away honeydew from area where hosts are no longer available. In contrast, in the case of parasitoids that exploit contact cues produced by the walking traces of the host, kairomones often are hexane soluble, suggesting that cuticular lipids may be involved (Howard and Flinn, 1990; Colazza et al., 2007; Rostás and Wölfling, 2009). Due to similar solubility properties, such compounds can be adsorbed and retained on the wax surface of leaves, and thus, can be easily perceived by the searching parasitoid. The parasitoid's response to contact cues also can be affected by experience which modifies the innate response to contact stimuli (Peri et al., 2006).

In order to better clarify the role of contact chemical cues in the host-parasitoid system *Trypoxylon politum* Say - *Melittobia digitata* Dahms, a series of bioassays were developed to investigate: (1) if *M. digitata* exploits contact kairomones from the host prepupa and/or host by-products, the cocoon, and the meconium; (2) the solubility and the chemistry of the kairomones; and (3) if experience affects the parasitoid's response to contact cues.

Methods and Materials

Insect Cultures Parasitoid wasps of the species *Melittobia digitata* were reared on naked prepupae of *T. politum* from stock cultures maintained in the Entomology Research Laboratory of Texas A&M University's Department of Entomology. Wasp cultures were kept in an incubator at 25°C and 70% RH in total darkness. Only macropterous (long-winged) females less than 5 d-old were used in the experiments. Because of their age, and since they were taken directly from cultures previous to their use in the bioassays, the wasps were presumably mated. About 1 hr before bioassays, wasps were transferred individually to size 1 gelatine capsules (vol: 0.5 ml; diam: 6.63 mm) and tested only once.

Hosts Prepupae of *Trypoxylon politum* Say were obtained from nests collected in 2009 (HW 47, West of Bryan, Texas

N 30° 36′, W 96° 24′) and kept in a refrigerator (<12°C) to maintain the prepupae in diapause for eventual use as hosts of *Melittobia* wasps. Previous to use in these bioassays, every cocoon was opened gently at the subtruncate cap (Cross et al., 1975) making a small hole in order to check if each host prepupa was healthy and not parasitized.

Open Arena Preparation Bioassays with *M. digitata* females were conducted in an open arena consisting of a rectangular filter paper sheet (120×140 mm). At the center of the arena, a circle with 58 mm diam. (2,642 mm^2, about 15% of the entire arena) was treated with the stimuli, while the rest of the filter paper was left untreated. We performed bioassays by using a flat surface in order to avoid any possible bias due to physical cues, since shape has been also shown to have a role in the host acceptance of *M. digitata* (Cooperband and Vinson, 2000). Each arena was used for testing five female wasps; it was then discarded and replaced with a new one. For each different experiment, the following preparation procedure used for bioassays was performed:

(1) *In vivo prepupa, cocoon, and meconium*. *Trypoxylon politum* prepupae are found protected by cocoons inside cells of mud nests. Each cell chamber contains a single cocoon/host (Fig. 1). Collected hosts were divided into three parts and tested separately: 1) prepupa; 2) empty cocoon; 3) dried meconium originally released by the mature *T. politum* larvae, and easily scraped from the bottom and inside the cocoon. The portion of cocoon to which the meconium was in direct contact was discarded and not used in the experiments to avoid any possible contamination.

(2) *Hexane and acetone host-by product extracts*. Hexane and acetone extracts were prepared for the following host-by products and tested separately: a) five cocoons with meconium but without prepupae inside (cocoon + meconium); b) five cocoons with no prepupae inside and meconium scraped out. The bottom part of the cocoon that was directly in contact with the meconium was not used in order to avoid any possible contamination with chemicals located on the meconium surface (cocoon only); c) five dried meconium pieces scraped from cocoons (meconium only).

Since *in vivo* bioassays (see Results) indicated that the prepupa of *T. politum* did not elicit a response to *M. digitata* wasps, we did not test the prepupa extract.

The cocoon + meconium, cocoon only or meconium only were placed in a 15 ml-glass vial and extracted with 10 ml of hexane or acetone (Sigma-Aldrich) at 28°C for 2 hr. After removal of host by-products, the resulting extracts were evaporated under a gentle nitrogen stream and redissolved in 200 µl of hexane or acetone. After each extract was chemically analyzed, it was stored at −18°C until bioassayed.

(3) *Reconstructed Blend of Straight-chain Carboxylic Acids*. The wasps responded strongly to apolar extracts (see Results). Thus, we investigated only the chemical composition of hexane extracts. Additionally, since the parasitoid's responses to cocoon and meconium hexane extracts were similar, and because of the high chemical similarities among identified compounds of the cocoon and meconium (see results and Table 1), we decided to use a solution containing an average quantity of each compound present in the cocoon crude extracts. This was done by reconstructing the hexane extract with a mixture of standard carboxylic acids, normalizing the concentration of the carboxylic acids found in crude extract to oleic acid, the most abundant compound. The stock of chemicals was prepared as a hexane solution containing (in mg/l): $C_{18:1}$(1000), $C_{18:2}$(618), C_{18}(369), C_{16}(294), C_8(262), $C_{16:1}$(125), C_{14}(124), C_7(94), C_6(90), C_9(30), C_{12}(17), (Sigma-Aldrich) (numbers given in brackets: see Table 1). The working solution was obtained by using 10 ml stock solution and diluting it to 100 ml. The reconstructed blend was stored at −18°C until bioassayed.

Open Arena Bioassay A single parasitoid female was gently introduced inside the lid of a small Petri dish (diam=35 mm) that was placed with the open end over the center of the treated area of the arena, thereby allowing the wasp to acclimatize. After 1 min and verification that the wasp was in direct contact with the arena, the lid was removed and observations of the unrestrained wasps' behavior were recorded. Recording stopped when the wasp walked off of the arena or after 10 min. For each experiment, the following bioassay procedure was used.

(1) *In vivo prepupa, cocoon, and meconium.* Prepupae, empty cocoons, or only meconium (5 of each) were gently rubbed in the center of the filter paper for about 5 min in order to contaminate the area homogenously with chemicals. Plain filter paper arenas served as controls.

(2) *Hexane and acetone host-by product extracts.* Hexane and acetone extracts of the cocoon + meconium, cocoon only, meconium only were tested at doses of 5 insect equivalent (IE) by pipetting 200 µl extract onto the central area of the arena. The 5 IE dosage was applied by repetitive pipetting of about 50 µl solution, and allowing the solvent to evaporate between repetitions. Filter paper in which the central area was treated with 200 µl of hexane or acetone was used as a control. The solvent was left to evaporate completely (~5 min.) before observation started.

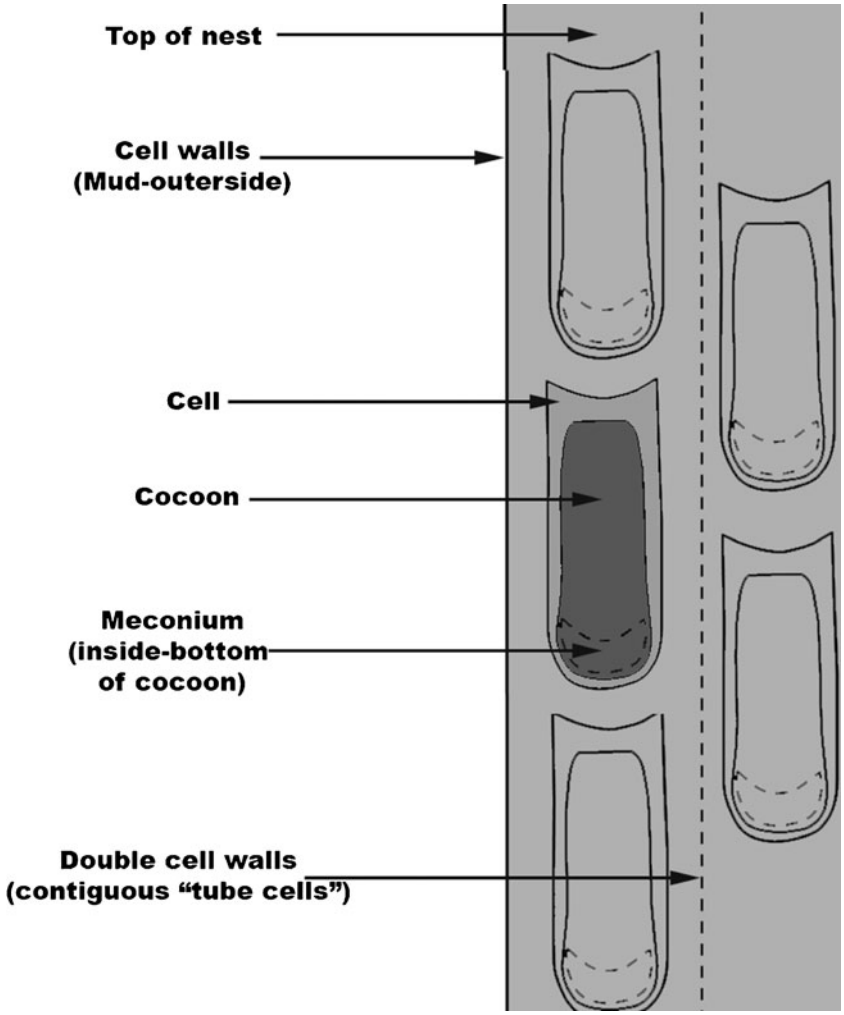


Fig. 1 Line drawing of *Trypoxylon politum* wasp mud nest showing the typical position of nest cells and the wasp cocoons inside. The dried meconium is shown inside the cocoons (in *broken lines*)

(3) *Reconstructed blend of straight-chain carboxylic acids*. Five IE of the reconstructed carboxylic acid blend of cocoon were tested by pipetting 20 µl of a hexane solution containing 0.1 mg/ml of the most abundant component ($C_{18:1}$) and proportional amounts of the minor components onto the treatment areas. Filter paper with the central area treated with 20 µl hexane served as control.

The walking pattern of *M. digitata* was observed using a video camera (EyeCom, PRO-9000) connected to a screen (Symphonic, 19-inch) in order to monitor the wasp away from the experimental arena and to avoid any possible disturbance effect of the observer on the behavior of the wasp. Meanwhile, data was digitalized and processed by using XBug, a video tracking and motion analysis system. The parameters computed by the system that best defined and described the parasitoid's behaviors were: (1) total residence time (sec); (2) average linear speed (mm/sec); (3) average angular speed (°/sec); (4) tortuosity index. This index ranges from 0 to 1, with 0 indicating a completely linear track, and 1 the maximum tortuosity. It was computed as follows: $T=1\text{-}mp/tl$; where T, tortuosity index; mp, maximum projection of the track over the generic line in the plan; and tl the total length of the track. All parameters were processed only for the central area and not for the total arena because we assumed that data from the treated area better characterized the behavior of the searching wasps stimulated by contact chemicals. To minimize cues from the room, the walls were painted black, and the experimental chamber was equipped with red fluorescent light (provided by 8, 40-W fluorescent bulbs covered with red tube guards) in order to keep visibility in the room in accordance to parameters previously established by González et al. (1985) and Matthews et al. (1985). For experiments (1) and (2), 25 replicates were performed, while for experiment (3), 40 replicates were performed. Experiments were carried out in a completely randomized design performing five replicates per experiment/day. The room was maintained at a temperature of 20°C and a RH of 60%. All bioassays were conducted from 10:00 to 12:00 and from 14:00 to 16:00.

Table 1 Mean amounts (± SE) of compounds identified from the cocoon and meconium of *Trypoxylon politum.* and their relative abundance (%)

Peak #	Compound[a]	Rt[b]	Amount (ng)		Relative abundance (%)		*t*-test[d]
			Meconium[c]	Cocoon[c]	Meconium	Cocoon	
1	Hexanoic (=caproic) acid	5.82	108.99±19.56	34.66±6.08	0.019	0.030	*
2	Heptanoic acid	8.49	142.49±29.00	36.31±4.79	0.024	0.031	*
3	Octanoic (=caprylic) acid	10.62	713.37±121.92	101.00±18.51	0.122	0.087	*
4	Nonanoic acid	12.36	66.52±15.48	11.61±2.47	0.011	0.010	*
5	Decanoic (=capric) acid	13.96	38.39±3.58	ND	0.007	ND	
6	Dodecanoic (=lauric) acid	16.73	25.22±7.99	6.52±1.87	0.004	0.006	*
7	Tetradecanoic (=myristic) acid	19.18	122.44±38.70	47.80±30.05	0.021	0.041	ns
8	(*Z*)-9-Hexadecenoic (=palmitoleic) acid	21.19	201.49±70.12	48.46±13.01	0.034	0.041	*
9	Hexadecanoic (=palmitic) acid	21.38	497.02±150.34	113.66±22.18	0.085	0.097	*
10	(*Z*,*Z*)-9,12-Octadecadienoic (=linoleic) acid	23.25	1320.48±453.60	239.13±88.91	0.225	0.205	*
11	(*Z*)-9-Octadecenoic (=oleic) acid	23.41	1786.54±554.19	386.70±105.35	0.305	0.331	*
12	Octadecanoic (=stearic) acid	23.73	843.88±228.91	142.73±24.32	0.144	0.122	*

[a] Carboxylic acids were first converted into methyl ester derivatives and then identified by comparison of retention times and mass spectra with authentic standards

[b] Rt retention time

[c] Hexane extracted compounds are equivalent to 1 meconium and 1 cocoon, respectively

[d] The amount of each carboxylic acid present in both cocoon and meconium hexane extracts was compared by using a *t*-test ($N=10$) *$P<0.05$; *ns* not significant; *ND* not detected

Gas Chromatography GC analyses were performed on a Hewlett Packard 6890 system with 7683 injector. One μl of each sample was injected onto a 0.32 mm×25 m SGE BP1 column in splitless mode. A temperature program of 50°C was set for 5 min., then 220°C at 10°/min. (5 min). Helium carrier gas at 20 psig was used. Ten samples in hexane of each type (cocoon and meconium) obtained as previously described in section (2) of the "open arena preparation procedure" were used for analysis. Mass spectral data were acquired on a Thermoelectron Trace GC2000 quadrupole GC-MS instrument. The attached GC unit was a Trace GC Ultra (Finnegan) equipped with a 0.25 mm×25 m SGE BP1 column operated at constant flow of 1 ml/min, using the same temperature gradient. Spectra were acquired in total ion current mode over a range of 40–500 amu with 70 eV electron impact ionization. Peak analysis was by comparison of acquired mass spectral data with that in the NIST 2001 libraries. Samples of all tentatively identified acids and of their methyl esters were purchased from Sigma-Aldrich chemical company, and retention time and mass spectra of these compounds were used for confirmation. Samples of acids then were prepared for bioassay. Due to poor peak symmetry of carboxylic acids, methyl ester derivatives were used for quantification. To obtain the methyl esters, 100 μl of crude insect derived extract for analysis, or 100 μl of a fatty acid mixture containing 1–10 μg fatty acids for standards were placed in a 2 ml HP screw cap vial, and 100 μl at 10% of BF3/MeOH were added to the vial, which was placed in heater block at 60° for 30 min., removing to shake well at 10 min. intervals. Then, 1 ml of water and 800 μl of hexane were added, shaking well and allowing layers to separate. The upper layer was removed carefully with a disposable pipette and filtered through anhydrous sodium sulfate, around 2 cm in a disposable pipette held in place by a small plug of Kimwipes, collecting the filtrate in a 2 ml crimp cap vial.

Detection Limits The limit of detection (LOD) of 1 ng/μl was determined by measuring progressively more dilute concentrations of the identified methyl esters until a signal-to-noise ratio of 3:1 was reached. Means and standard error for amounts of each compound in each sample type were calculated based on ten replicates (Table 1).

Effect of Experience To test if there was any influence of the experience elicited by exposing wasps to host by-products (cocoon or meconium) or to the host itself (prepupa) on the parasitoid's arrestment response, wasps less than 5 d-old were isolated together with the source of the stimulus into small glass vials (1 dram) with tight cotton stoppers and kept for 24 hr prior to bioassays; in the case of exposure to prepupa, *M. digitata* initiated host feeding and oviposition after 2–6 hr; such parasitoids were experienced with respect to oviposition. The response of cocoon-experienced wasps, meconium-experienced wasps, or prepupa-experienced wasps were tested subsequently on

filter paper arena contaminated, as *in vivo* bioassays, with contact chemicals of the cocoon, meconium, or prepupa, respectively. As controls, we used naïve females of the same age that were tested in arenas contaminated with chemicals of the cocoon, meconium, or prepupa, respectively, in order to make paired comparison between experienced and naïve wasps. For each set of bioassays 25 replicates were performed.

Statistical Analysis Data were not significantly different from a normal distribution (Shapiro-Wilk test $P=0.05$). Data from the *in vivo* bioassays, from host by-product extracts, and from reconstructed blends of carboxylic acids were analyzed by one-way ANOVA followed by Tukey's HSD *post hoc* test for comparisons between means. Comparisons between naïve and experienced female wasps were done by using a *t*-test. The amounts of each carboxylic acid extracted from cocoon and meconium also were compared by using a *t*-test. All statistical analyses were performed by using Statistica 6.0 for Windows (StatSoft Inc., Tulsa, OK, USA).

Results

For all experiments with control conditions, wasp residence time in the arena was short, and the tortuosity index was low. Control wasps spent little time in the central area, exploring at most only small sections of the arena's surface (Fig. 2a). With the different treatment conditions, in those experiments that elicited a behavioral response of wasps, there was increased residence time in the central treated area compared to controls. In general, wasps spent more time within the contaminated (treated) area and performed a more convoluted locomotion pattern, thus resulting in a more complete exploration of the arena (=arrestment response) characterized by a high tortuosity index. Females often were observed turning back to reenter the treated area after exceeding its boundaries (Fig. 2b).

In vivo Prepupa, Cocoon, and Meconium Residence time in the central area was higher in the arena contaminated with host by-products compared to those contaminated with either the host prepupa or the control ($F=10.59$; $df=3,96$; $P<0.001$) (Fig. 3a). In fact, no significant difference was found between time (mean ± SE) spent by wasps on the prepupa-contaminated arena (59.91±22.11 sec) and time spent on the control (27.21±6.40 sec). Linear speed was lower on patches contaminated by cocoon and by meconium compared to patches contaminated by prepupa or the control ($F=46.15$; $df=3,96$; $P<0.001$) (Fig. 3b). Higher values of angular speed of parasitoid were found in the arena contaminated by prepupa compared to those contaminated by host by-products or control ($F=21.23$; $df=3,96$; $P<0.001$)

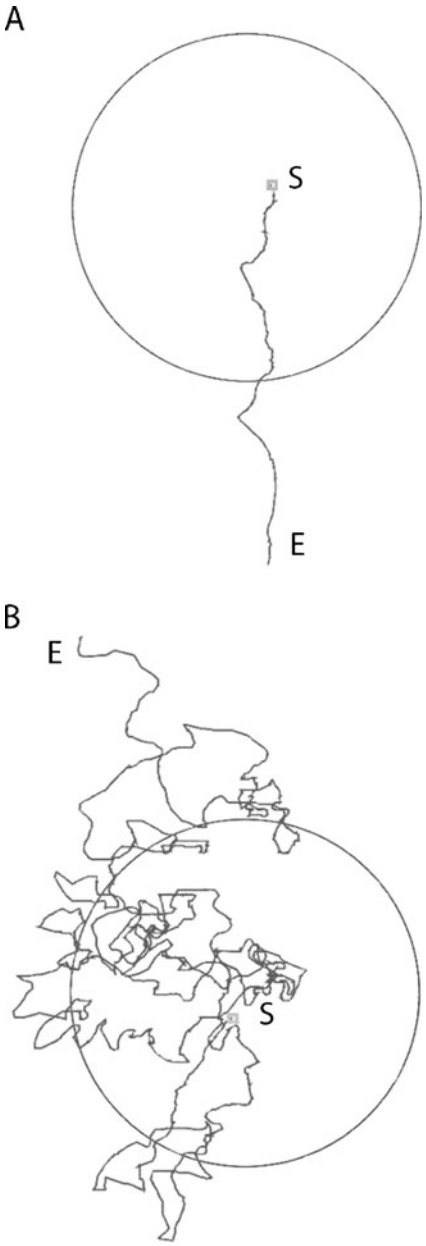


Fig. 2 Examples of two recorded paths (A+B) of *Melittobia digitata* in an open arena consisting of a rectangular filter paper sheet (120× 140 mm) (central circle area 58 mm diam.). **a** Untreated central area. **b** Central area rubbed with *Trypoxylon politum* cocoons. *S* start; *E* end

(Fig. 3c). Tortuosity was higher on the patches contaminated by host by-products compared with the control, and intermediate in those contaminated by prepupa ($F=30.12$; $df=3,96$; $P<0.001$) (Fig. 2d).

Hexane Host By-Product Extracts Residence time was higher in the arenas contaminated with cocoon + meconium extract, cocoon extract, and meconium extract compared to the control ($F=4.06$; $df=3,96$; $P=0.009$) (Fig. 4a). Linear speed was lower on patches contaminated with cocoon +

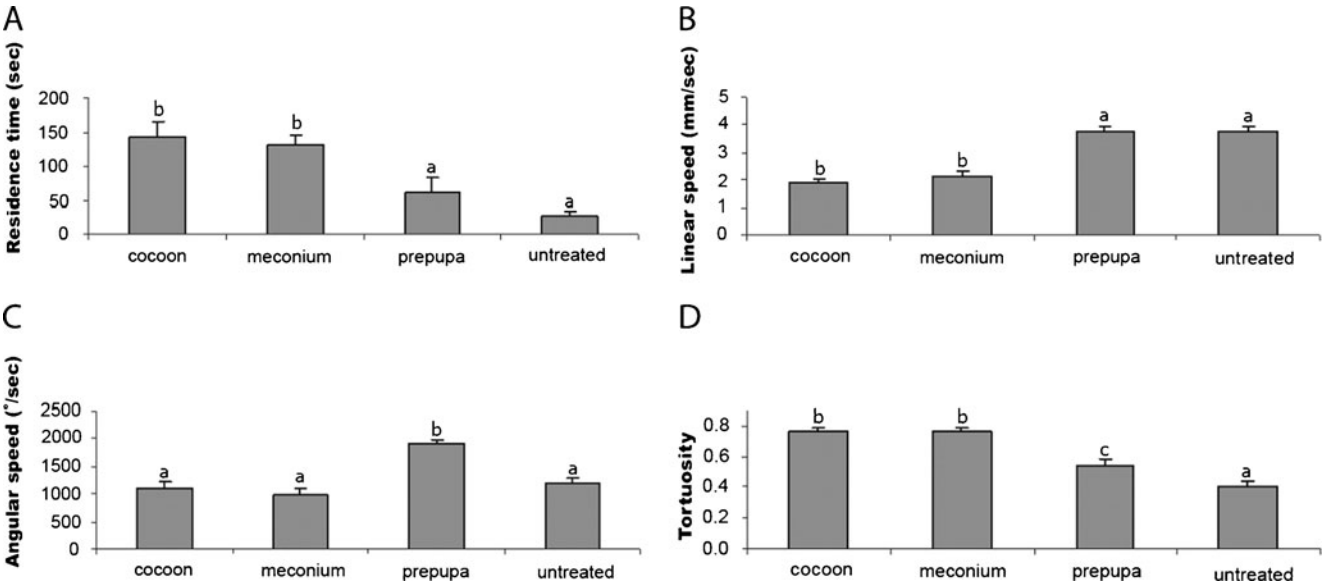


Fig. 3 Various measures (mean±SE) of *Melittobia digitata* responses to contact chemicals from rubbed cocoon, rubbed meconium, and rubbed prepupa of *Trypoxylon politum*. Untreated arena served as control. Columns with the same letter are not significantly different at $P<0.05$ (ANOVA, Tukey tests)

meconium extract compared to the other tested conditions or the control ($F=6.67$; $df=3,96$; $P<0.001$) (Fig. 4b). Higher values of angular speed of parasitoid were found in the arena contaminated by cocoon extract compared to the other tested conditions or control ($F=9.49$; $df=3,96$; $P<0.001$) (Fig. 4c). Tortuosity was higher within the patches contaminated by extract of cocoon + meconium, cocoon, and meconium compared to the control ($F=7.30$; $df=3,96$; $P<0.001$) (Fig. 4d).

Acetone Host By-Product Extracts Central area residence time was higher for arenas contaminated with cocoon + meconium extract compared to the other host by-product extracts or control ($F=5.96$; $df=3,96$; $P<0.001$) (Fig. $4a_1$). There were no significant differences in the linear speed of the parasitoid on host by products contaminated patches over the control ($F=2.05$; $df=3,96$; $P=0.112$) (Fig. $4b_1$). Higher values of the angular speed were found for arenas treated with extract of cocoon + meconium or cocoon compared to those contaminated by meconium extract or controls ($F=14.95$; $df=3,96$; $P<0.001$) (Fig. $4c_1$). Tortuosity was higher on the patches contaminated by extract of cocoon + meconium or cocoon compared to control ($F=8.92$; $df=3,96$; $P<0.001$) (Fig. $4d_1$). However, no statistically significant differences were found between cocoon extract and meconium extract nor between meconium extract and control (Fig. 4d).

Reconstructed Blend of Straight-chain Carboxylic Acids The mix of linear carboxylic acids (test) elicited a weak but significant response in terms of arena residence time compared to the control ($F=9.32$; $df=1,78$; $P=0.003$). In fact, time (mean ± SE) spent by the wasps on the test arenas was 76.72±13.77 sec, while the time spent on the control arenas was 32.29±4.81 sec.

Chemical Analyses Twelve detected compounds, all linear carboxylic acids, were identified in the host by-product hexane extracts of *T. politum*. The five most abundant compounds were octanoic (cocoon[c] = 8.7%; meconium [m] = 12.2%), palmitic (9.7%c; 8.5%m), linoleic (20.5%c; 22.5%m), oleic (33.1%c; 30.5%m),and stearic acid (12.2%c; 14.4%m) representing 84.2% and 88.1% of the total carboxylic extract of cocoon and meconium, respectively. Qualitative differences were found, since decanoic acid was only detected in small quantities from meconium extract, and quantitative differences also were found because, for all carboxylic acids, excluding myristic acid, the relative amount was significantly higher for meconium extract compared to the cocoon extract; however, the compounds were present in similar ratios (Table 1) (Fig. 5).

Effect of Experience Central arena residence time was not significantly influenced by exposure of wasps to host by-products (cocoon: $t=0.677$; $df=48$; $P=0.501$; meconium: $t=0.174$; $df=48$; $P=0.862$) nor to exposure to the host itself (prepupa: $t=-0.878$; $df=48$; $P=0.384$); even though experienced parasitoids increased the time (mean ± SE) spent on an arena contaminated by prepupa chemicals (naïve = 59.91±22.11 sec; experienced = 93.85±31.70 sec), no statistical differences were found between values of naïve vs. experienced wasps; similar values were found for the time

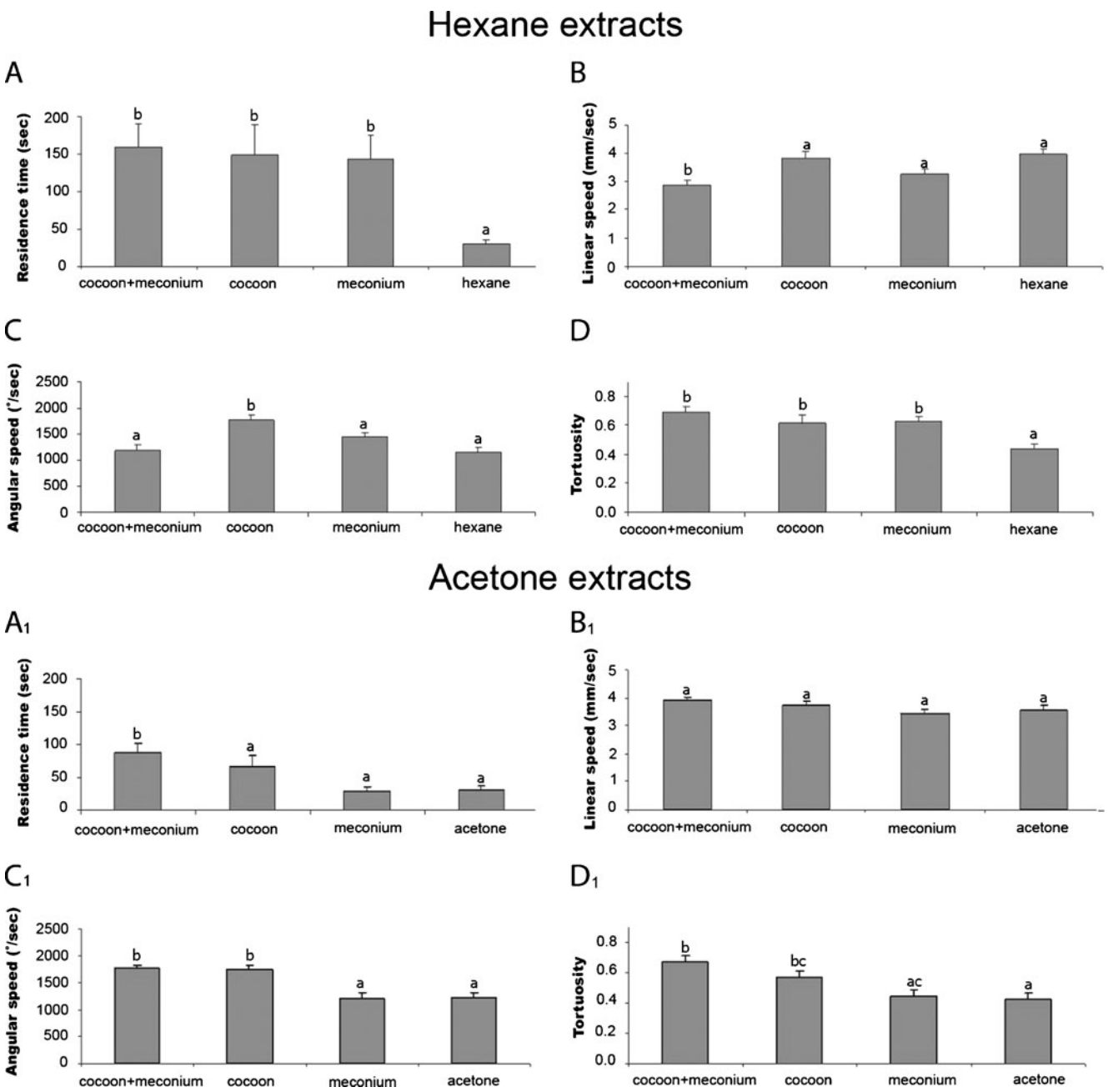


Fig. 4 Various measures of *Melittobia digitata* responses to hexane extracts (*above*) and acetone extracts (*below*) of cocoon + meconium, cocoon only, and meconium only of *Trypoxylon politum*. Arena treated with hexane or acetone served as control. Columns with the same letter are not significantly different at $P<0.05$ (ANOVA, Tukey tests)

spent by naïve and experienced wasps on patches contaminated with host by-products (cocoon: naïve = 143.86± 22.50 sec; experienced = 120.87±25.40 sec; meconium: naïve = 132.49±12.88 sec; experienced = 129.38±12.27 sec).

Discussion

The results of the *in vivo* bioassays showed clearly that *M. digitata* was arrested by contact kairomones from the host by-products (cocoon, meconium) providing the first evidence that contact cues are exploited by *Melittobia* wasps. Surprisingly, the host stage attacked, *T. politum* prepupa, did not induce the parasitoid to spend significantly more time in an arena treated with chemicals in their exocuticle when compared to the control. Contact chemicals located in the host cuticle are cues always associated with the host, but *M. digitata* seems not to exploit them during the host selection process. Such behavior may be explained by considering that the host selection process is divided into a

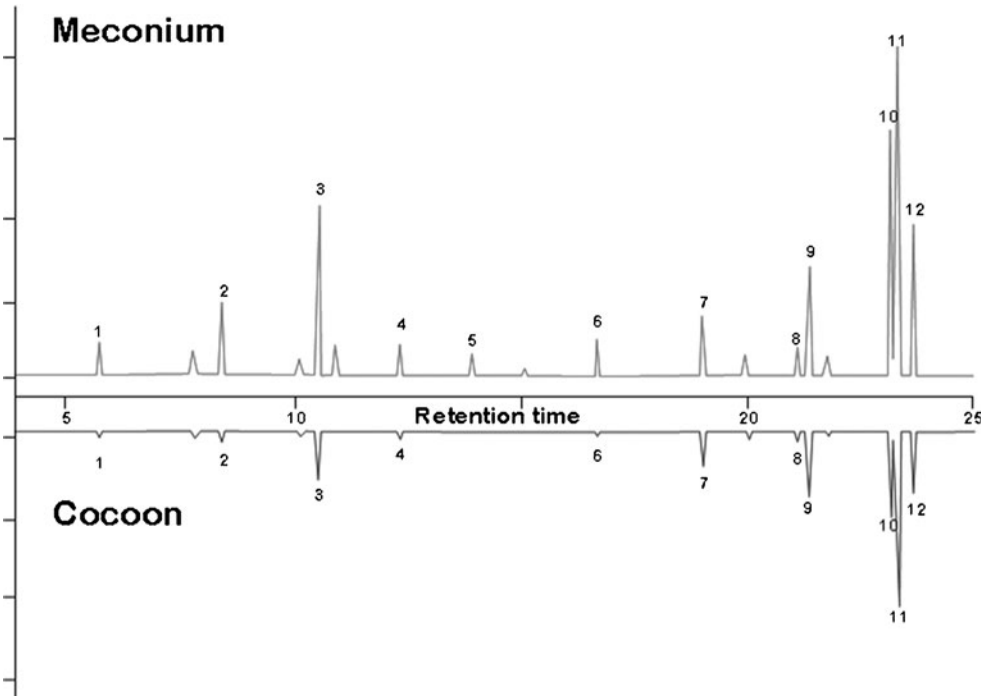


Fig. 5 Representative gas chromatograms of compounds extracted from cocoon and meconium of *Trypoxylon politum* with hexane. For peak identities, see Table 1; "Methods and Materials" describe analysis conditions

series of hierarchical steps in which each step is influenced by the previous one: the parasitoid, under natural condition, is expected to find a prepupa after having found a cocoon and contacted the meconium; hence, the lack of occurrence of the host by-product cues may explain the non-response to contact chemicals from the prepupa since cocoon and meconium are always associated with the presence of a suitable host. In addition, it also is possible that physical or tactile cues play an important role when parasitoids first contact a potential host as shown by Cooperband and Vinson (2000); chemical information could be secondary or redundant to physical cues.

The bioassays conducted with polar and non-polar extracts revealed that *M. digitata* responded strongly to hexane extract of cocoon + meconium, cocoon only, and meconium only, suggesting that the nature of the kairomones is mainly non-polar soluble. Since the response of the parasitoid was similar when the hexane extract of the cocoon or the hexane extract of the meconium was tested, we assume that contact kairomones of these host-by products share similarity. Indeed, the cocoon and the meconium have the same origin, and they are both produced by the mature larva just prior to its transformation to the prepupa (which is the suitable stage for parasitoid offspring development). GC and GC/MS analysis of cocoon and meconium extracts showed the presence of linear carboxylic acids (C_6–C_{18}). Cocoon and meconium extracts contained almost identical compounds in similar proportion (the most abundant compounds were: octanoic acid; hexadecanoic acid; (*Z*,*Z*)-9,12-octadecadienoic acid; (*Z*)-9-octadecenoic acid; octadecanoic acid).

Fatty acids are common compounds in nature that play roles in various ecological contexts, including the host location process of some parasitoids (Renou et al., 1992; Horikoshi et al., 1997). However, when the reconstructed blend of fatty acids was tested, *M. digitata* showed a weak response compared with the control; hence, even if carboxylic acids play a role, probably some other unidentified compounds present in small quantities are also involved. Many studies show that synthetic blends have only low activity when compared to the activity of crude extracts, thus supporting the presumed high complexity of contact kairomones. For example, the egg parasitoid *Trissolcus basalis* (Wollaston) weakly responded to a reconstructed blend of straight-chain hydrocarbons compared to hexane extracts of other host cuticular lipids (Colazza et al., 2007); the larval parasitoid *Cotesia marginiventris* Cresson weakly responded to reconstructed blends of linear *n*-alkenes compared with hexane extracts of the host larvae footprint and of the larval ventral cuticle (Rostás and Wölfling, 2009).

Melittobia digitata can successfully parasitize a large variety of hosts that belong to different insect orders. In general, the less specialized a parasitoid is, the more likely that experience can affect its behavior (Vet and Dicke, 1992). In our experiment, no difference was found between experienced and naïve wasps regardless if they were experienced on a cocoon, on meconium, or on prepupa. In the latter case, *M. digitata* was able to host feed and even oviposit on the host, resulting in rewards experienced by the wasps, which could act as strong reinforcing stimuli. We expected that prepupa-experienced wasps would spend significantly more time compared with naïve wasps when

tested on filter papers arena contaminated with contact cues of the prepupae. However, this did not occur. Unlike many other parasitoids that attack and parasitize multiple hosts, thereby gaining repeated oviposition experience, *Melittobia* females usually locate and parasitize (and/or superparasitize) only a single host; thus, prior host experience would not be selectively favored. Silva-Torres et al. (2005b) reared *M. digitata* on 3 different hosts: *T. politum*, *Sarcophaga bullata* (Parker), and *Megachile rotundata* (Fabricius), and found that the female's natal host did not influence host choice. This suggests that pre-imaginal experience is irrelevant for *M. digitata* wasps as well.

Cusumano et al. (2010) stressed the importance of volatile cues emitted by host by-products of *T. politum* in the host location process of *M. digitata* macropterous females. The results of our study indicate that once *M. digitata* is within the host cell, host acceptance is mediated by chemicals of low volatility that arise following host meconium release and cocoon construction.

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New Sex Pheromone Blend for the Lightbrown Apple Moth, *Epiphyas postvittana*

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Abstract The composition of the sex pheromone gland of the lightbrown apple moth, *Epiphyas postvittana* (Walker) was re-investigated. In addition to the two previously identified compounds, (*E*)-11-tetradecenyl acetate (E11-14Ac) and (*E,E*)-9,11-tetradecadienyl acetate (E9E11-14Ac), seven additional candidate pheromone compounds were identified: (*E*)-11-tetradecen-1-ol (E11-14OH), tetradecyl acetate, hexadecanal, (*E*)-11-hexadecenyl acetate (E11-16Ac), hexadecyl acetate, octadecanal, and octadecyl acetate. Gas chromatographic-electroantennographic detection analysis showed that only four (of the nine) compounds (E11-14OH, E11-14Ac, E9E11-14Ac, and E11-16Ac) elicited electrophysiological responses. When either E11-14OH or E11-16Ac were tested at various ratios in three-component blends with both E11-14Ac and E9E11-14Ac, no increase in trap catch was observed compared to that to the previously identified binary blend. However, when these two compounds (1% E11-14OH and 0.5% E11-16Ac) were both added to E11-14Ac and E9E11-14Ac, trap catch was roughly double that to the previously identified binary blend alone. The new four-component blend should be more sensitive for detecting this economically important insect, especially in low population areas (i.e., in newly invaded habitats). In addition, this new blend may enhance pheromone control of this pest, through approaches such as mating disruption, lure and kill, and mass trapping.

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Key Words Lightbrown apple moth · *Epiphyas postvittana* · Sex pheromone · Pheromone identification · Monitoring and control · Lepidoptera · Tortricidae

Introduction

The lightbrown apple moth (LBAM), *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae) is an important pest of horticulture in its native Australia and in introduced habitats of New Zealand, USA, and UK (Suckling and Brockerhoff, 2010). The endemic Australian genus, *Epiphyas*, contains approximately 60 species (Hitchcock, 2009). LBAM larvae typically feed on leaves and the fruit surface by webbing a leaf to a fruit to create a protected shelter (Lo et al., 2000). This causes unsightly blemishes on fruit and can lead to secondary disease development, causing rots, in crops such as grapes (Bailey et al., 1996). LBAM is known as a pest primarily of tree fruits, including apples, pears, citrus, peaches, nectarines, apricots, vines, and berryfruit, but also, to a lesser extent, of forestry, vegetable, and flower crops (Wearing et al., 1991). The sex pheromone of LBAM was initially identified as a two-component blend of (*E*)-11-tetradecenyl acetate (E11-14Ac) and (*E*,*E*)-9,11-tetradecadienyl acetate (E9E11-14Ac) (Bellas et al., 1983), in a ratio of 100:5, respectively. Further work on the sex pheromone (Foster and Roelofs, 1990) found tetradecyl acetate (14Ac) in glands at about 10% of the major component. However, Muggleston and Foster (1989) found no evidence for a behavioral role for this compound in wind tunnel assays.

During work on the quantification of the sex pheromone of this insect, as part of a study on fitness of irradiated insects, several additional potential pheromone compounds were found. Since most moth species use multi-components

blends for sexual communication (El-Sayed, 2011), we wondered whether any of these compounds were involved in male attraction? Therefore, we undertook to re-investigate the chemistry of the female sex pheromone gland comprehensively, and to field test any additional potential pheromone compounds identified. As result, we identified a new four-component blend that attracts more males than the previously identified binary blend. This identification is particularly timely, as LBAM continues to expand beyond its native habitat and was recently confirmed in the USA, Sweden, and other parts of Europe; this new blend could help in the eradication of, or management efforts for, this pest.

Methods and Materials

Insects A colony of LBAM was established and maintained at Mt Albert Research Centre (Auckland). The insects were sexed as pupae, with adult males allowed to emerge in isolation from females. Males and females were kept at 22± 2°C, 18:6 hr L:D, and were provided with water. All insects were 2–3 d old at time of testing.

Chemicals All compounds used as authentic standards in the chromatographic analyses or field trapping experiments were >97% pure by gas chromatographic (GC) analysis, and were stored at −80°C until used. Samples of E11-14Ac, E9E11-14Ac, (*E*)-11-tetradecen-1-ol (E11-14OH), hexadecanal (16Ald), (*E*)-11-hexadecenyl acetate (E11-16Ac), hexadecyl acetate (16Ac), and octadecyl acetate (18Ac) were purchased from Plant Research International, Wageningen, The Netherlands. Tetradecyl acetate (14Ac) was purchased from Bedoukian Research Inc., Connecticut, USA, and octadecanal (18Ald) was synthesized at Plant & Food Research. Each blend of synthetic compounds, in 200 µl of *n*-hexane GR (Merck Ltd, New Zealand), was applied to the large "well" of a red rubber septum (West Pharmaceutical Services, Kearney, NE, USA). The solvent was allowed to evaporate in a fume hood, and the septa were stored in heat-sealed foil bags at −20°C until ready for use in field trapping experiments.

Pheromone Gland Extraction The sex pheromone glands of 2 to 3-d-old calling females (>10 females) were removed during the first 2 hr of the scotophase and extracted in ca 20 µl of hexane for 5–10 min. Analysis of the gland extracts was performed by using gas chromatography-electroantennogram detection (GC-EAD) or gas chromatography–mass spectrometry (GC-MS) as described below. Quantification of each compound in the gland extract was conducted using an external standard method (Scott, 1995), with known amounts (0.1, 1.0, 10.0, and 100.0 ng) of each compound injected into the GC-MS. The results were subjected to linear regression analysis (SAS Institute Inc., 1998) to determine the relationship between amount of compound injected and peak area of the mass chromatogram.

GC-MS Analysis Gland extracts and synthetic chemicals were analyzed on a Saturn 2200 GC-MS (Varian Walnut Creek, CA, USA), using an ionization voltage of 70 eV and a mass range of 30 to 650, equipped with splitless injection and two capillary columns: a non-polar 30 m×0.25 mm i.d.× 0.5 µm, VF5-MS and a polar 30 m×0.25 mm i.d.×0.5 µm, VF23-MS (both Factor Four, Varian Inc, USA). For both columns, the oven was programmed from 80°C (held for 1 min.) to 240°C at 10°C min^{-1}. Compounds were identified by comparison of retention times and mass spectra with those of synthetic compounds.

GC-EAD Coupled GC-EAD analyses of gland extracts were conducted on a Varian 3800 GC equipped with a flame ionization detector (FID), a splitless injector, and a 30 m×0.25 mm i.d.×0.5 µm, VF5-MS capillary column. The oven temperature was programmed from 80°C (held for 1 min) to 220°C at 10°C.min^{-1}, and then held for 13 min. Helium was used as carrier gas. The column effluent was split 1:1 (by a Graph pack—3D/2 crosspiece splitter; Gerstel GmbH & Co, KG, Germany) between the FID and EAD apparatus. Antennal depolarization was detected by a high-resistance EAD Probe, Signal Interface Box, Type ID-02 and Intelligent Data Acquisition Controller, Type IDAC-02 (Syntech, Hilversum, The Netherlands). Antennae from 2 to 3-d-old males were excised at the base and attached to silver electrodes housed in saline-filled glass electrodes. Ten antennal preparations were used in the analyses.

Field Trapping Experiments

Optimization of the Ratio of E11-14OH and E11-16Ac The previously identified binary blend (Bellas et al., 1983) of E11-14Ac and E9E11-14Ac (0.1:0.005 mg) was supplemented with different amounts (0, 0.001, 0.002, and 0.005 mg) of either of the two EAD-active compounds, E11-14OH and E11-16Ac, and tested on rubber septa in the field using red delta traps, made of plastic corflute, with an adhesive-coated base (The New Zealand Institute for Plant & Food Research Ltd, Auckland) (Suckling and Shaw, 1992). An additional experiment was conducted to test lower amounts of E11-16Ac, using the two-component blend (E11-14Ac: E9E11-14Ac; 0.1: 0.005 mg) supplemented with varying amounts of E11-16Ac (0, 0.0001, 0.0005, and 0.001 mg). Traps baited with each of the pheromone blends were placed in five rows, with five replicates of each

treatment in a randomized block design. Traps were positioned 1.7 m above the ground in a tree, and were spaced 20 m apart in each row. Each treatment was assigned randomly to a tree within a row of trees. Septa were placed in the center of the sticky base. Sticky bases were replaced weekly during the experimental period. A trap with a blank lure was used as a control.

Other Multi-component Blends The attractiveness of the following blends was tested: a nine-component blend (E11-14OH, E11-14Ac, 14Ac, E9E11-14Ac, E11-16Ac, 16Ald, 16Ac, 18Ald, and 18Ac, in a ratio of 0.001, 0.1, 0.005, 0.005, 0.0005, 0.005, 0.005, 0.01, and 0.005 mg, respectively), a four-component blend (E11-14OH, E11-14Ac, E9E11-14Ac, E11-16Ac, in a ratio of 0.001, 0.1, 0.005, and 0.0005 mg, respectively), and the binary blend (E11-14Ac, E9E11-14Ac, in a ratio of 0.1 and 0.005 mg, respectively). Traps baited with a single, caged female were used as positive controls, and traps with a blank lure as negative controls.

A final experiment was conducted to eliminate non-attractive components of the blends, particularly saturated aldehydes, which were considered unlikely to be attractive because of the lack of electrophysiological responses. The following blends were tested: a seven-component blend lacking the two saturated aldehydes (E11-14OH, E11-14Ac, 14Ac, E9E11-14Ac, E11-16Ac, 16Ac, and 18Ac in a ratio of 0.001, 0.1, 0.005, 0.005, 0.0005, 0.005, and 0.005 mg, respectively), a six-component blend lacking the three saturated acetates (E11-14OH, E11-14Ac, E9E11-14Ac, E11-16Ac, 16Ald, and 18Ald in a ratio of 0.001, 0.1, 0.005, 0.0005, 0.005, and 0.01 mg respectively), a four-component blend lacking the saturated aldehydes and acetates (E11-14OH, E11-14Ac, E9E11-14Ac, E11-16Ac, in a ratio of 0.001, 0.1, 0.005, and 0.0005 mg, respectively), and the binary blend (E11-14Ac, E9E11-14Ac, in a ratio of 0.1 and 0.005 mg, respectively). A trap baited with a blank lure was used as a control. The experimental design and protocol of these last two experiments were identical to the other experiments, except that 14 replicates were used in the first experiment and 10 replicates in the second experiment.

Data Analysis

The effect of treatment on the mean number of LBAM males captured was tested using ANOVA (SAS Institute Inc., 1998), after variances were stabilized using the $\sqrt{x}$ transformation. Significantly different treatment means were identified *post-hoc*, using Fisher's Protected Least Significant Difference Test at the 5% level (SAS Institute Inc., 1998).

Results

Chemical Identification Chemical analyses of the pheromone gland extracts of female *E. postvittana* showed seven additional minor compounds that had a pheromone-type structure (Table 1). The first compound had the diagnostic ions *m/z* 55, 67, 81, 95, 109, and 123, and eluted earlier than the main pheromone compound on a non-polar column, suggesting that it was either a saturated aldehyde or monounsaturated alcohol or acetate. The lack of *m/z* 61 excluded the acetate functionality, suggesting that it would be either a saturated aldehyde or monounsaturated alcohol. The retention time and Kovat's retention indices (KI) of this compound matched E11-14OH on both polar and non-polar columns. Four of the new compounds had the diagnostic *m/z* 61, characteristic of acetates. Three of these compounds had the diagnostic *m/z* 69, 83, 97, 111, and 125, suggesting saturated acetates. Retention times and KIs of these compounds matched those of 14Ac, 16Ac, and 18Ac on both polar and non-polar columns. The fourth acetate had the diagnostic *m/z* 67, 81, 95, 109, 123, and 222, suggesting a monounsaturated 16-carbon acetate. Retention times and KIs of this compound matched E11-16Ac on both polar and non-polar columns. The final two structures had the diagnostic *m/z* 55, 67, 81, 95, 109, and 123, suggesting

Table 1 Normalized ratios and retention indices of the candidate pheromone compounds found in sex pheromone gland extract of female lightbrown apple moth, *Epiphyas postvittana*

Compound	ng/female[a]	Ratio (%)	Non Polar VF5-MS	Polar VF23-MS
(*E*)-11-tetradecen-1-ol	0.01	0.1	1,679	2,310
(*E*)-11-tetradecenyl acetate	9.18	100	1,804	2,254
Tetradecyl acetate	0.52	5.7	1,809	2,204
Hexadecanal	0.58	6.3	1,824	2,320
(*E*,*E*)-9,11-tetradecadienyl acetate	0.48	7.1	1,868	2,480
(*E*)-11-hexadecenyl acetate	0.05	0.7	1,999	2,466
Hexadecyl acetate	0.42	4.6	2,010	2,422
Octaadecanal	0.76	8.3	2,028	2,552
Octadecyl acetate	0.04	0.4	2,108	2,641

[a] $N=5$

they were either saturated aldehydes or monounsaturated alcohols. Retention times and KIs of these compounds matched 16Ald and 18Ald on both polar and non-polar columns.

GC-EAD Analysis Analysis of gland extracts by GC-EAD revealed that four compounds consistently elicited responses from male moth antennae. E11-14Ac elicited the strongest EAD response, followed by E9E11-14Ac, E11-14OH, and E11-16Ac (Fig. 1).

Optimization of the Ratio of E11-14OH and E11-16Ac The addition of 1, 2, or 5% E11-14OH to the two-component blend of E11-14Ac and E9E11-14Ac did not result in an increase in the number of LBAM males caught in the traps (Fig. 2). Similarly, the addition of 1, 2 or 5% E11-16Ac to the binary blend did not result in an increase in the number of LBAM males caught (Fig. 3, top). It was evident that the number of males caught declined as the amount of E11-16Ac increased (Fig. 3, top). Therefore, we conducted another experiment testing lower amounts of E11-16Ac (0. 0.1 or 0.5 or 1%). However, there was no difference in male catch at any of the ratios tested (Fig. 3, bottom).

Other Multi-component Blends In the first experiment that tested other multicomponent blends, there was an effect of blend on the number of males caught in the trap ($F_{5,78}$=9.8, P<0.001). The four-component blend caught the greatest number of males, with the mean catch greater than that of the binary blend (Fig. 4). No difference was observed between mean catch to the nine-component blend and that to the two-component blend. Caged females caught fewer males than the binary blend (Fig. 4). In the final experiment, traps baited with the three multi-component (4, 6, or 7) blends captured significantly more males than traps baited with the binary blend. There was no difference in trap catch among the 4, 6, or 7-component blends (Fig. 5), showing that the saturated aldehydes or acetates had no detectable effect on trap catch.

Discussion

Many moth species utilize multi-component pheromones for sexual communication. When species share common pheromone components, the composition of multi-component blends is critical for species specificity. As there are many species in the Australian genus *Epiphyas* (Hitchcock, 2009), with E11-14Ac and E9E11-14Ac found in a number of them (Horak et al., 1988), it is likely that multi-component blends are critical for ensuring specificity of response within the genus.

Analysis of LBAM sex pheromone gland extracts confirmed the presence of seven additional (E11-14Ac and E9E11-14Ac) compounds: two monounsaturated compounds (E11-14OH and E11-16Ac), three saturated acetates (14Ac, 16Ac, and 18Ac), and two saturated aldehydes (16Ald and 18Ald). Apart from 14Ac (Foster and Roelofs, 1990), this is the first report of the presence of these compounds in the sex pheromone gland of LBAM females. GC-EAD analysis demonstrated that, of these seven compounds, only E11-14OH and E11-16Ac elicited an EAD response. Field trapping studies identified a new four-component pheromone blend for LBAM, consisting of the two previously identified components, E11-14Ac and E9E11-14Ac, plus E11-14OH and E11-16Ac. This blend elicited roughly double the catch of males compared to that of traps baited with the previously identified binary blend. E11-14OH has been reported as a pheromone component of many tortricid moth species, for example, *Choristoneura orae* (Gray et al., 1984), and, *C. parallela* (Neal et al.,

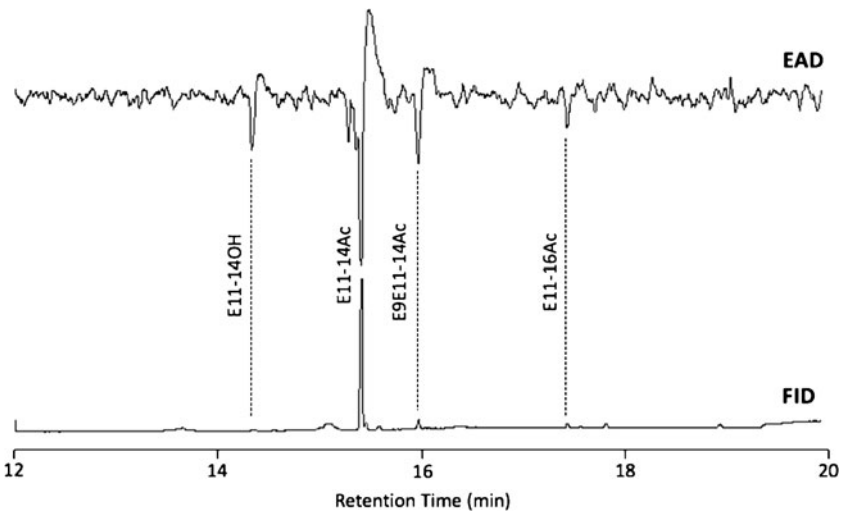


Fig. 1 Coupled gas chromatographic-electroantennographic detection responses of male *Epiphyas postvittana* to female pheromone gland extract. *EAD* electroantennogram detection; *FID* flame ionization detection; *E11-14OH* (*E*)-11-tetradecen-1-ol; *E11-14Ac* (*E*)-11-tetradecenyl acetate; *E9E11-14Ac* (*E*, *E*)-9, 11-tetradecadienyl acetate; *E11-16Ac* (*E*)-11-hexadecenyl acetate

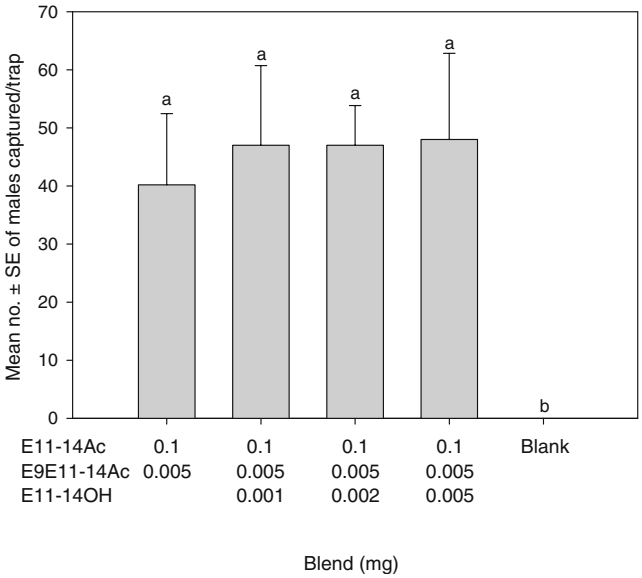


Fig. 2 Mean catch (± SE) of *Epiphyas postvittana* males caught in traps baited with a three-component blend containing a constant 0.1: 0.005 mg proportion of (*E*)-11-tetradecenyl acetate (E11-14Ac): (*E*, *E*)-9, 11-tetradecadienyl acetate (E9E11-14Ac) plus varying amounts of (*E*)-11-tetradecenol (E11-14OH). The experiment was conducted from 18 February 2010 to 4 March 2010 in apple orchards at Lincoln, New Zealand. A total of 911 male moths were captured. Treatments labeled with the same letter were not significantly different at $P<0.05$

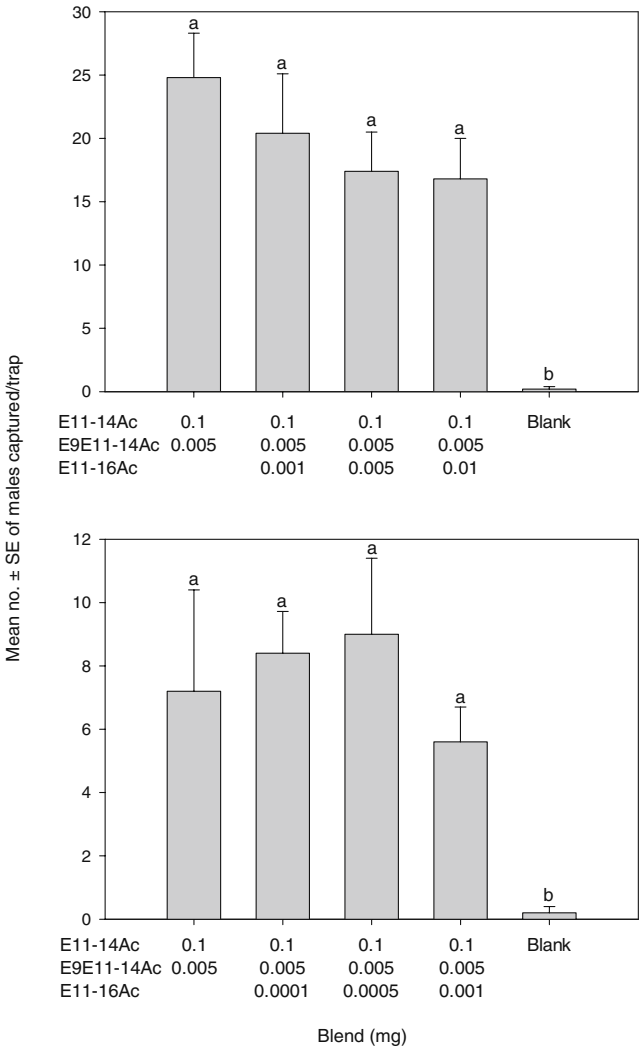


Fig. 3 Mean catch (± SE) of *Epiphyas postvittana* males in traps baited with a three-component blend containing a constant 0.1: 0.005 mg proportion of (*E*)-11-tetradecenyl acetate (E11-14Ac): (*E*, *E*)-9, 11-tetradecadienyl acetate (E9E11-14Ac) plus varying amounts of (*E*)-11-hexadecenyl acetate (E11-16Ac). The two experiments were conducted from 11 March 2010 to 12 April 2010 in apple orchards at Lincoln, New Zealand. A total of 556 male moths were captured. Treatments labeled with the same letter were not significantly different at $P<0.05$

1982). In contrast, E11-16Ac has not been reported previously as a sex pheromone component of tortricid moth species, but is commonly found in species of Crambidae, Noctuidae, and Saturniidae (El-Sayed, 2011).

The addition of the saturated aldehydes, 16Ald and 18Ald, or the saturated acetates, 14Ac, 16Ac, and 18Ac, to the new quaternary blend had no effect on male catch. Previous work had shown that the addition of different ratios of 14Ac to the binary blend did not enhance the number of males arriving at the odor source (Muggleston and Foster, 1989). In light of our work, this is not surprising, as 14Ac did not elicit an EAD response. The saturated acetates, 14Ac, 16Ac, and 18Ac, commonly are found in the sex pheromone glands of species of tortricid moths, and have been reported to be part of the sex pheromone blend in a few cases. In contrast, the two saturated aldehydes have not been reported to be sex pheromone components of a tortricid moth (El-Sayed, 2011).

Bellas et al. (1983) identified only E11-14Ac and E9E11-14Ac in the sex pheromone gland of female *E. postvittana*, and demonstrated that a 100:5 ratio was optimal for male attraction. The inability of these authors (Bellas et al., 1983) to identify the two new minor compounds reported here is likely due to improved sensitivity of today's GC-MS systems, and perhaps the lack of use of a GC-EAD.

In a study of sex pheromone gland content in LBAM females, the amount of E9E11-14Ac to E11-14Ac, varied from 2.2% to 11% (Muggleston and Foster, 1989). Male LBAM responded to a wide range of ratios of these two components, from 1:10 to 1:50 (Bellas et al., 1983). There could be similar variation in production of, and response to, the new components, as indicated by the fact that addition of different ratios of E11-14OH or E11-16Ac to the binary blend had little positive or negative effect on trap catch. In many species of moths, minor compounds invoke either a peak response to a tight ratio, or similar responses over a wide range of ratios. For example, in the European

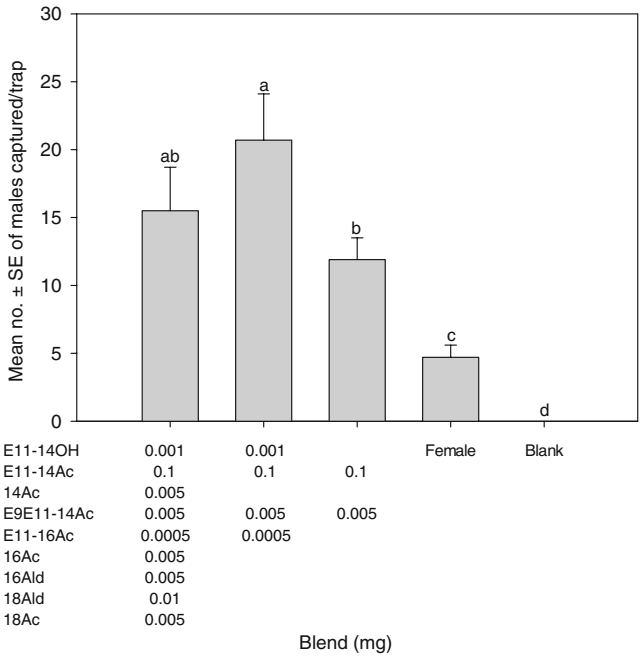


Fig. 4 Mean catch (± SE) of *Epiphyas postvittana* males in traps baited with four different pheromone blends: a nine-component blend with all compounds identified in the gland; a four-component blend with all compounds that elicited an electroantennographic response; and the previously identified binary blend. Caged virgin females were used as positive controls and blanks as negative controls. The experiment was conducted from 30 April 2010 to 15 May 2010 in apple orchards at Lincoln, New Zealand. A total of 744 male moths were captured. Treatments labeled with the same letter were not significantly different at $P<0.05$. E11-14OH = (*E*)-11-tetradecenol; E11-14Ac = (*E*)-11-tetradecenyl acetate; 14Ac = tetradecyl acetate; E9E11-14Ac = (*E*, *E*)-9, 11-tetradecadienyl acetate; E11-16Ac = (*E*)-11-hexadecenyl acetate; 16Ac = hexadecyl acetate; 16Ald = hexadecanal; 18Ald = octadecanal; 18Ac = octadecyl acetate

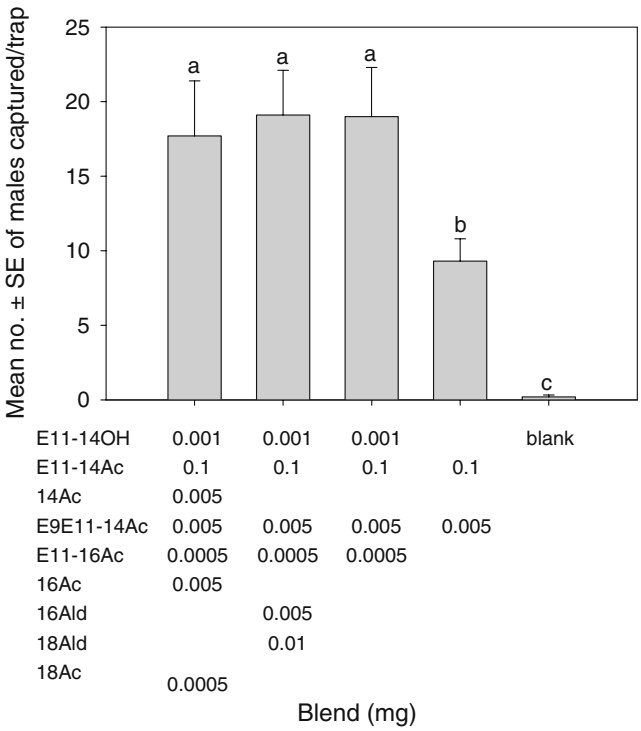


Fig. 5 Mean catch (± SE) of *Epiphyas postvittana* males in traps baited with four different pheromone blends: a seven-component blend containing all compounds identified in the gland except the saturated aldehydes; a six-component blend containing all compounds identified in the gland except the saturated acetates; a four-component blend containing all compounds that elicited an electroantennographic response; the previously identified binary blend; a blank was the negative control. The experiment was conducted from 11 to 25 May 2010 in apple orchards at Lincoln, New Zealand. A total of 659 male moths were captured. Treatments labeled with the same letter were not significantly different at $P<0.05$. E11-14OH = (*E*)-11-tetradecenol; E11-14Ac = (*E*)-11-tetradecenyl acetate; 14Ac = tetradecyl acetate; E9E11-14Ac = (*E*, *E*)-9, 11-tetradecadienyl acetate; E11-16Ac = (*E*)-11-hexadecenyl acetate; 16Ac = hexadecyl acetate; 16Ald = hexadecanal; 18Ald = octadecanal; 18Ac = octadecyl acetate

grapevine moth, *Lobesia botrana*, the two minor components, (*E*,*Z*)-7,9-dodecadienol and (*Z*)-9-dodecenyl acetate, elicited peak attraction at 5% and 1% amounts (of the major component), respectively (El-Sayed et al., 1999). In tortricid moths, such as *Grapholita molesta* (Baker et al., 1981; Linn and Roelofs, 1983), *Adoxophyes orana* (Guerin et al., 1986), *Cacoecimorpha pronubana* (Witzgall, 1990), and *Spilonota ocellana* (Witzgall et al., 1991), males responded to a wide range of ratios of the minor components.

Traps baited with our new blend caught more males than traps baited with caged virgins. Indeed, even the binary blend elicited greater trap catches than did caged females. In previous work, catches from traps baited with three females were similar to those from traps baited with rubber septa loaded with 105 μg of the binary blend (Suckling and Brockerhoff, 1999). The relatively low catch in traps baited with virgins in the current work could be due to the quality of the females used or to adverse weather and light conditions during the experiment, which could have caused females to release pheromone for restricted periods.

LBAM is a major pest of tree fruits, berryfruit, forestry, vegetable, and flower crops in New Zealand and Australia. Recently, this insect expanded its range to California, Sweden, and other parts in Europe. In the initial stages of invasion, the population density of a given invasive species is usually low and hard to detect. The availability of a more potent sex pheromone blend for LBAM should make it easier to detect this insect at low population densities. This may make an eradication strategy, using both mass trapping and lure and kill more feasible (El-Sayed et al., 2006, 2009). We anticipate that the improved pheromone blend will be a valuable tool in the eradication and pest management efforts against this pest.

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The Level of Major Urinary Proteins is Socially Regulated in Wild *Mus musculus musculus*

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Abstract Major urinary proteins (MUPs) are highly polymorphic proteins that have been shown to perform several important functions in the chemical communication of the house mouse, *Mus musculus*. Production of these proteins in C57Bl/6 females is cyclic, reaching the maximum just before the beginning of estrus. Social environment is an important factor that increases MUP production in both sexes. We examined responsiveness of MUP production to social stimuli in wild mice, *Mus musculus musculus*. The direction of change of MUP production in males depended on the sex of the stimulus animal. Males up-regulated MUP production when caged with a female, but down-regulated MUP production when caged with a male. Down-regulation was more pronounced in males that were defeated in a male–male encounter. Females responded to a male's presence with a decrease in MUP production. We conclude that social modulation of MUP production is specific and, in coordination with other mechanisms, facilitates adjustment of the animal's odor profile to different social contexts. Our results also suggest that in males, MUPs may play an important role in advertizing the male's quality to females. Furthermore, we highlight the importance of analyzing data corrected with creatinine, which show MUP production on the (post)translational level as well as raw data (non-corrected with creatinine), which represent actual concentrations of MUPs in the urine.

Key Words Chemical communication · House mouse · Major urinary protein (MUP) · *Mus musculus musculus* · Polyacrylamide gel electrophoresis · (SDS PAGE) · Social modulation

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Introduction

Major urinary proteins (MUPs) are lipocalins that are excreted in substantial amounts in the urine of the house mouse, *Mus musculus* L. (Flower, 1996). In their internal binding pocket, they bind and transport diverse sets of volatile pheromone ligands (Bacchini et al., 1992; Robertson et al., 1993; Novotny et al., 1999). The key role of these proteins is the stabilization of mouse chemical signals by slowing down the evaporation of their pheromonal ligands. Hurst et al. (1998) proved that pheromones bound to MUPs are released more gradually and for a considerably longer period from a deposited scent mark, whereas free pheromones are, due to their high volatility, lost within a few hours.

However, MUPs have been shown to perform additional functions in intra-specific chemical communication. These functions are primarily facilitated by extreme MUP polymorphism, which has been observed in wild mice, principally in *Mus musculus domesticus* Rutty (Beynon and Hurst, 2003). Different individuals of this subspecies have been shown to express different combinations of alleles from the MUP gene cluster that is located on chromosome 4 and contains as many as 21 genes (Krauter et al., 1982; Stopkova et al., 2009). In populations of *M. m. domesticus* from Great Britain (Robertson et al., 1997) and Switzerland (Rusu et al., 2008), the combinatorial nature of MUP expression results in the occurrence of multiple distinct MUP profiles composed of different isoforms. Due to their high variability, genetic inheritance, and presumptive stability, these MUP profiles are suitable

candidates for the signalling of a variety of genetically determined characteristics such as kinship (Sherborne et al., 2007), gender (Armstrong et al., 2005), or heterozygosity (Thom et al., 2008). Moreover, the variability of MUP profiles in *M. m. domesticus* from Great Britain is so substantial that each individual possesses a unique MUP chemical signature that has been reported to be employed in identity recognition (Cheetham et al., 2007).

The diversity of MUP roles in chemical communication has been highlighted with the finding that male MUPs can directly induce, without their pheromonal ligands, a physiological or behavioral response in a recipient. In juvenile females, they accelerate the onset of puberty (Mucignat-Caretta et al., 1995), and in adult females that are held in crowded living conditions, they increase the amount of ova (Morè, 2006). MUPs also act directly in intrasexual communication where they trigger aggressive behavior between males (Chamero et al., 2007). Several results suggest that MUPs mediate their pheromonal effect through activation of an accessory olfactory pathway (Morè, 2006; Chamero et al., 2007).

There is complex and dynamic regulation of MUP production that employs genetic and epigenetic mechanisms and several hormones, including testosterone, growth hormone, and thyroxine (Knopf et al., 1983; Johnson et al., 1995; Roemer et al., 1997). Testosterone is known to be primarily responsible for the high levels of MUPs in males when compared to females (Wicks, 1941; Thung, 1956). In females, hormones may participate in the progression of the estrous cycle and influence the production of MUPs, thus inducing the fluctuation of MUP quantity in the course of the female reproductive cycle. This fluctuation with maximum MUP titer reached just at the beginning of estrus may be used to signal either directly or through the binding and release of MUP pheromonal ligands, a female's reproductive receptivity to potential mates (Stopka et al., 2007).

Stopka et al. (2007) showed that MUP production is up-regulated following an introduction of individuals of the opposite sex to singly caged C57BL/6 males and females. This increase of MUP quantity may reflect an enhanced need for communication in animals that are in social contact when compared to animals held in isolation. However, we hypothesized that the regulatory role of the social environment may be more specific. It would be advantageous for animals if MUP production is regulated differently in different social contexts, thus contributing to modulation in the production and release of an animals' chemical signal that should fulfill its roles in a variable social environment. Such social dependence has been reported, for example, for the MUP regulatory agent, testosterone, whose level is strongly affected by social interactions (Macrides et al., 1975; Van Oortmerssen et al., 1987; Oyegbile and Marler, 2005), and which in turn regulates production of several male pheromones known to enhance the attractiveness of a male and his urinary scent marks to females (Jemiolo et al., 1991; Ferkin et al., 1994; Zala et al., 2004).

Here, we explored the dependence of MUP quantity on different social conditions. Inbred mice, used in our previous study (Stopka et al., 2007), are descendants of excessive brother-sister breeding and exhibit atypical behavioral responses (Nevison et al., 2000; Lacey and Hurst, 2005). They express a limited number of MUPs that can substantially affect their roles in chemical communication (Nevison et al., 2003; Cheetham et al., 2009). Therefore, we decided to use wild, unrelated mice as experimental subjects. We examined the effects of cohabitation with individuals of the same or opposite sex on MUP quantity in the East-European house mouse, *Mus musculus musculus* L. Although past research has been focused primarily on *M. m. domesticus* and laboratory mice it has been shown that MUPs are also produced in high quantities in *M. m. musculus* (Sampsell and Held, 1985; Stopkova et al., 2007). The level of MUPs in males of this subspecies is even higher than in male *M. m. domesticus* (Stopkova et al., 2007). Moreover, a high level of sexual dimorphism indicates that the roles of MUPs in this subspecies may be different in males and females (Janotova and Stopka, 2009). Another aim of our study was to provide new information about MUP expression in *M. m. musculus*. We confirmed the modulatory effect of social environment on MUPs in *M. m. musculus*, and analyzed the variation of MUP production in males that were exposed to social stimuli from other males with respect to their competitive abilities.

Methods and Materials

Subjects of Study and Experimental Setup The mice (*Mus musculus musculus*, 30 males, 10 females) used were caught at different sites within the Czech Republic (Sedlecko [50º14′11.611″N, 12º57′28.763″E], Vintirov [50º14′3.795″, 12º43′16.715″E], Brandys [50º11′1.653″N, 14º39′49.804″E], Svetla Hora [50º2′52.555″N, 17º24′0.948″E], and Znojmo [48º50′48.596″N, 16º3′54.453″E]). Experiments were carried out in accordance with the European Communities' Council Directive of November 24, 1986 in the University Breeding Facility (Accreditation licence number: 1020/919/A/00). The experimental procedures were approved by the Czech Ministry of Agriculture (licence to P Stopka—No. CZU 514/01). Animals were kept on a 12:12 h light cycle with lights off at 1900 h. Water and food were provided *ad libitum*. We tested three experimental groups: females that were subjected to male stimuli (Females), males exposed to females (Males), and males exposed to other males (Male-

Male). All animals were adults and held in the laboratory for at least 1 mo before the beginning of the experiment. The experiment was divided into two parts, each lasting 10 d. In the first part (individual), all subjects were housed singly in cages containing clean bedding material (sawdust Lignocel 3/4, J. Rettenmaier & Söhne, Rosenberg, Germany). In the second part (social), subjects were paired and caged in compartmented enclosures, where they were separated from each other by a metal grid with circular openings (radius was 0.4 cm) allowing communication but suppressing direct contact. The experimental cages were supplied with fresh bedding at the beginning of the social part and were not cleaned until the end of the experiment (10 d). There were 10 pairs in each experimental group. In pairs, animals were unfamiliar to each other and captured from different sites. Males were paired with same-sex counterparts of similar weights.

Urine samples were collected every day for the duration of the experiment (both individual and social parts) in the beginning of the light phase by gentle bladder massage, and stored in polyethylene 1.5 ml Eppendorf tubes, and frozen at −20°C. All samples were analyzed within 1 mo from time of collection. We also checked the estrous stage of females daily. Vaginal smears were stained by using method of Pappenheim (1899) and analyzed under a light microscope.

The response of males in same-sex experimental pairs may be influenced by the social hierarchy, which should establish in the pair during the course of experiment. Therefore, after finishing the social part of the experiment, we allowed members of each pair to interact freely in a neutral arena for 5 min. In order to assess the social relationship between the males, we recorded the number and direction of attacks and submissive behavioral patterns such as crouching, freezing, or fleeing (Zielinski and Vandenbergh, 1993; D'Amato et al., 2001). When aggression escalated (the attacks from one male were continuous) or any of males showed visible wounds, the confrontation was interrupted immediately.

Protein Analysis Urinary proteins were separated by SDS-PAGE in the Mini-PROTEAN III Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA) according to a modified protocol from Laemmli (1970). Samples were defrosted, stirred, and centrifuged for 5 min at 16000 x *g*. The volume of 0.1–0.2 μl of male and 0.2–0.4 μl of female urine, respectively, contains enough protein to ensure optimal separation and accurate quantification of bands. To avoid pipetting such small amounts, which can lead to inaccuracy, we diluted 1 μl of urine with 9 μl of distilled water and loaded an appropriate amount of this solution onto a 10% Tris-Glycine gel. Moreover, to exclude potential loading error, we added 1000 ng (2 μl) of carbonic anhydrase as a marker for SDS-PAGE (29 kDa, Sigma-Aldrich, St. Louis, MO, USA) to each sample.

Gels were stained with Coomassie® G-250 (SimplyBlueTM Safe Stain, Invitrogen Life Technologies, Paisley, UK). Gel images were acquired by using a GS-800 Calibrated Densitometer. Denaturing polyacrylamide gel electrophoresis used to separate urinary proteins gave a well-resolved band with an approximate size of 18 kDa. This band represents MUPs, as was confirmed by western blotting (data not shown) with Goat Anti-Mouse MUP serum (YN-GMMUP, Accurate Chemical & Scientific Corporation, Westbury, NY, USA) as a primary antibody.

The concentration of MUPs in urine samples was determined by volumetric analysis of bands on polyacrylamide gels in Quantity One 1-D Analysis Software (Bio-Rad Laboratories). The volume of each band was defined as the sum of intensities of the pixels inside a defined boundary. Mean background intensity was subtracted from the acquired values.

The concentration of urinary proteins depends greatly upon the volume of excreted urine. To eliminate variability in the data set attributable to different urine dilutions between samples, we measured creatinine concentration by using LKreatinin kit (BioVendor, Brno, CR) and calculated the dilution coefficient kdil=(1/Cref)*Ccreat, (where Cref is the reference creatinine concentration and Ccreat is creatinine concentration of each sample). The reference value was defined as the highest concentration of all measured creatinine concentrations. Corrected volumes were obtained by dividing each MUP volume by the dilution coefficient.

Data Analysis The absolute concentration of MUPs represented by creatinine non-corrected values is an important parameter that should be included in the analysis even though it changes as the urine mark dries. Therefore, we performed a statistical analysis on both sets of data, corrected by creatinine as well as non-corrected by creatinine. Creatinine-corrected data are referred to as MUP production, whereas data analyzed without creatinine correction are denoted as MUP concentration.

For analysis of social modulation, we used samples collected on d 1 of the 10 d individual part. From the social part, samples were collected 24 h after the animals were paired, which is based on our previous experience where we showed that 24 h was sufficient to trigger socially dependent changes in MUP production. To analyze the differences in MUP quantity between the sexes, Student's two tailed *t*-test was performed on the data from the individual experimental setup. These data also were used to examine individual variation. However, due to insufficient volume of some urinary samples, we could not perform a creatinine assay on all samples (none of the samples included in social modulation analysis belonged to these low-volume samples). Therefore, because of the incompleteness of creatinine corrected data, only creatinine non-corrected data (representing concentration) was used to

analyze individual variation in MUP quantity. Since there were big differences in MUP levels among individuals we calculated ratio scores by dividing the data obtained in the social experimental setup by the data from the individual experimental setup prior to statistical analysis of social modulation. The ratio scores were transformed to the natural logarithm scale to remove non-linearity. Statistical analyses were performed in STATISTICA 6.0 (StatSoft Inc., Tulsa, OK,USA 2001). We used *One-Way ANOVA* and *post-hoc* Tukey test to analyze individual differences and the effect of social treatment. The effect of social hierarchy on the responses of males in male-male pairs was tested by using *Repeated Measures ANOVA*. To assess changes in MUP quantity in females during the estrous cycle *Nested-Design ANOVA* was used. The results were considered significant at a critical level of $\alpha=0.05$.

Results

Sexual Dimorphism and Individual Differences Prior to analysis of samples from mice held under social conditions, we aimed to assess individual variability and the level of sexual dimorphism of MUP quantity in experimental animals. Big differences among individuals were revealed in males (*One-Way ANOVA*; $F_{(29,270)}=32.23$, $P<0.001$) and females ($F_{(9,90)}=18.13$, $P<0.001$; Fig. 1). For this reason, further analyses were conducted on the ratio scores of data from "individual" and "social" parts of the experiment (see Methods). The variability among different localities was tested with male samples because they represented all trapping sites. However, no differences among the five localities were observed (*One-Way ANOVA*; $F_{(4,25)}=0.74$; $P=0.57$). The level of sexual dimorphism was significant in both concentration ($t=-3.74$, $d.f.=38$, $P<0.001$; $\text{mean}_{(\text{females})}=5.1$ mg/ml, s.e.$=\pm1.97$; $\text{mean}_{(\text{males})}=33.2$ mg/ml, s.e.$=\pm4.5$) and production of MUPs ($t=-4.43$, $d.f.=38$, $P<0.001$; $\text{mean}_{(\text{females})}=9.1$ mg/ml, s.e.$=\pm3.65$; $\text{mean}_{(\text{males})}=80.5$ mg/ml, s.e.$=\pm12.61$).

Social Modulation Pairing of mice that were previously caged as individuals with same or different sex conspecifics resulted in a significant change of MUP production in all treatment groups (Fig. 2a). When we analyzed creatinine non-corrected data (representing MUP concentration), a significant change was only found in females that were exposed to the presence of a male. In the other two treatment groups, the confidence intervals overlapped with the zero line, which indicates that the effect of treatment was not significant (Fig. 2b).

Differences between groups were investigated by using *One-way ANOVA* followed by a *post-hoc* Tukey test. Differences in MUP production ($F_{(2,37)}=7.92$; $P=0.001$) and concentration ($F_{(2,37)}=6.58$; $P=0.004$) (Fig. 2) were significant, indicating that the groups reacted differently to the experimental treatment. Responses were dependent on the sex of both test and stimulus individual. Of the 10 test males, 8 showed up-regulation of MUP production when stimulated by the presence of a female. When a same-sex individual was introduced into the cage, down-regulation was observed (in 15 of the 20 males). Difference between the responses of the two male groups, Males (exposed to a female) and Male–Male, respectively, exposed to different sex conspecifics was significant (*Tukey*, $P=0.007$). On the level of concentration, the difference between the responses of these two groups was not signficant ($P>0.05$). Females (8 of 10) reacted to the male's presence with decrease in MUP quantity. The response of this group was significantly different from the response of males stimulated by a female in both MUP production (*Tukey*, $P=0.002$) and concentration (*Tukey*, $P=0.003$).

The analysis of behavioral patterns of freely interacting males performed after finishing the experiment revealed a clear asymmetry of agonistic interactions that shows that the social hierarchy was established in all male-male pairs. In each pair, we were able to identify a male who initiated most attacks (winner) and apparently dominated the other member of the pair who displayed frequent submissive behaviors (loser; see Methods). The median of number of attacks (NA) for winners was 13.5 (quartiles:11.2; 14). The median NA for losers was 1 (quartiles: 0.25; 1.75). In one case, the encounter had to be interrupted after 3 min to prevent serious injury because of continuous attacks (at least 20 attacks) from one male, who was then considered to be the winner. Males significantly down-regulated MUP production when presented with same sex conspecific (*repeated measures ANOVA* $F_{(1,18)}=6.86$; $P=0.017$). The category of male (winner and loser) was also a significant factor influencing males' response ($F_{(1,18)}=4.67$; $P=0.044$) showing that winners expressed more protein than losers. Combination of both factors, treatment and status, did not reveal any significant effect, indicating that both tested male categories responded to the treatment equally ($F_{(1,18)}=0.56$; $P=0.46$), i.e., with down-regulation (Fig. 3). However, when the concentration of MUPs was analyzed, no significant result was found ($F_{(1,18)}=3.58$; $P=0.075$).

The difference in results obtained for MUP concentration and MUP expression in males indicates that urine dilution had changed due to experimental treatment. To confirm this, we analyzed creatinine concentration in the urine of tested animals. Urine of loser males became more concentrated when compared to winners in which no change of urinary concentration was apparent ($F_{(1,18)}=5.02$; $P=0.038$; Fig. 4). Thus, losers lowered MUP level, but MUP concentration in the urine remained unchanged.

In our experiment, an incidence of estrus was approximately two times more frequent in the second part of the experiment

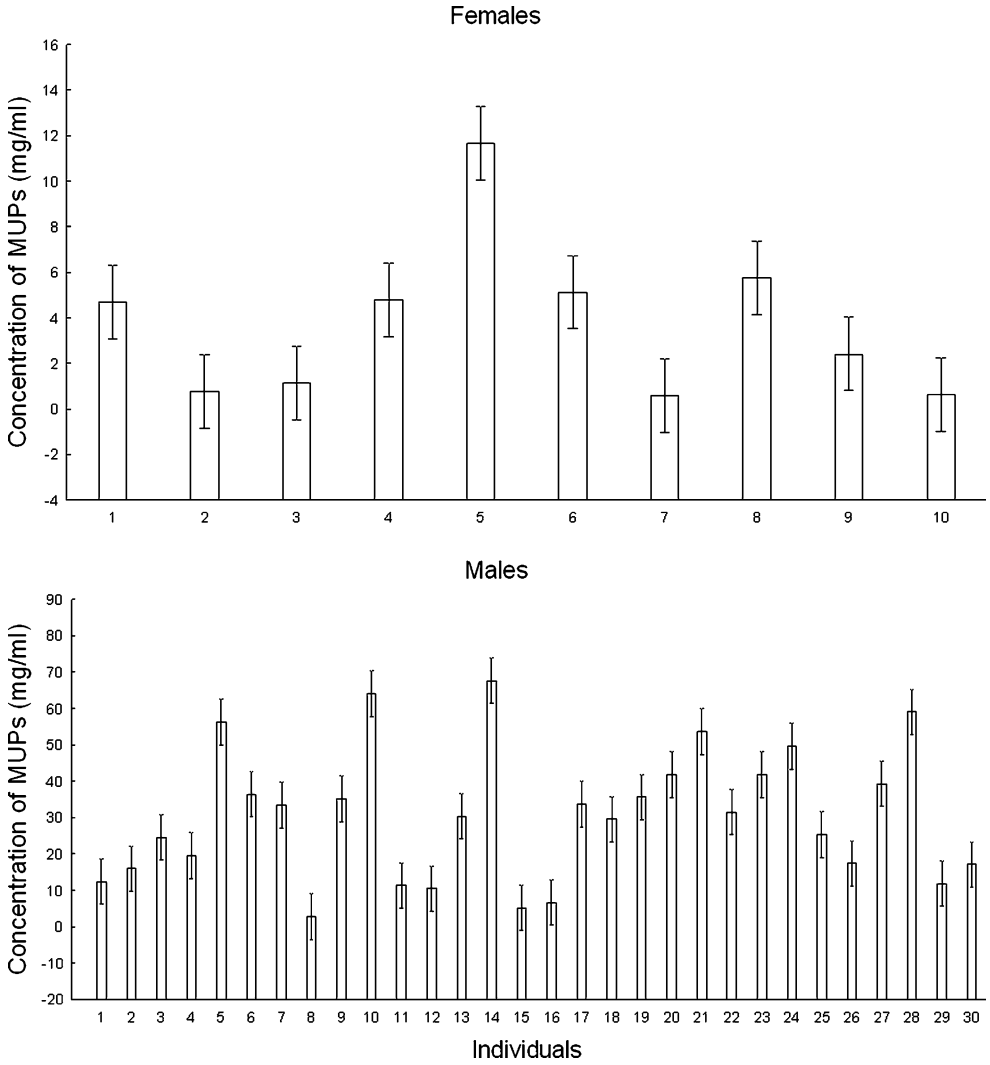


Fig. 1 Individual variation of major urinary protein concentration in male and female house mice, *Mus musculus musculus*. Bars represent 95% confidence intervals. $N_{(females)}=10$; $N_{(males)}=30$

(42 out of 100) when females were exposed to male stimuli than in the first, individual part (20 out of 100). In the social part, all females showed estrous cycling, while in the individual setup, three of females failed to display estrus. Because of it we did not assess fluctuation of MUP quantity in the course of the reproductive cycle in individually housed females. However, *Nested-design ANOVA* revealed that under social conditions, MUP production did not change significantly ($F_{(10,80)}=1.53$, $P=0.14$) during the estrous cycle.

Discussion

The variation of MUP production under individual and social housing conditions in laboratory mouse strain C57BL/6 has been reported by Stopka et al. (2007). Here, we extend this research result and provide evidence that this phenomenon is not restricted to laboratory mice but represents an intrinsic characteristic of MUP expression in a wild mouse species (*M. m. musculus*). Our results demonstrate that stimulation of an individual by the presence of conspecifics influences expression level of urinary MUPs, and that due to changes of urine dilution, this may not necessarily be manifested on the level of urinary MUP concentration. Moreover, we showed that the direction of MUP production change (i.e., either up- or down-regulation) differs according to the sex of both tested and stimulus animal. This indicates that observed variation of MUPs is not a nonspecific response that should solely ensure a sufficient strength of chemical signals in animals that have been isolated and subsequently subjected to social contact with conspecifics. Instead, it seems to be a specific response that facilitates a modification of the animal's urinary odor profile in different social conditions. The mechanism of this modification may be either direct, utilizing pheromonal potency of MUPs (Mucignat-Caretta et al., 1995; Morè, 2006; Chamero et al., 2007) or indirect, operating through modulation of ligand evaporation by MUPs (Marie et al., 2001; Veggerby et al., 2001).

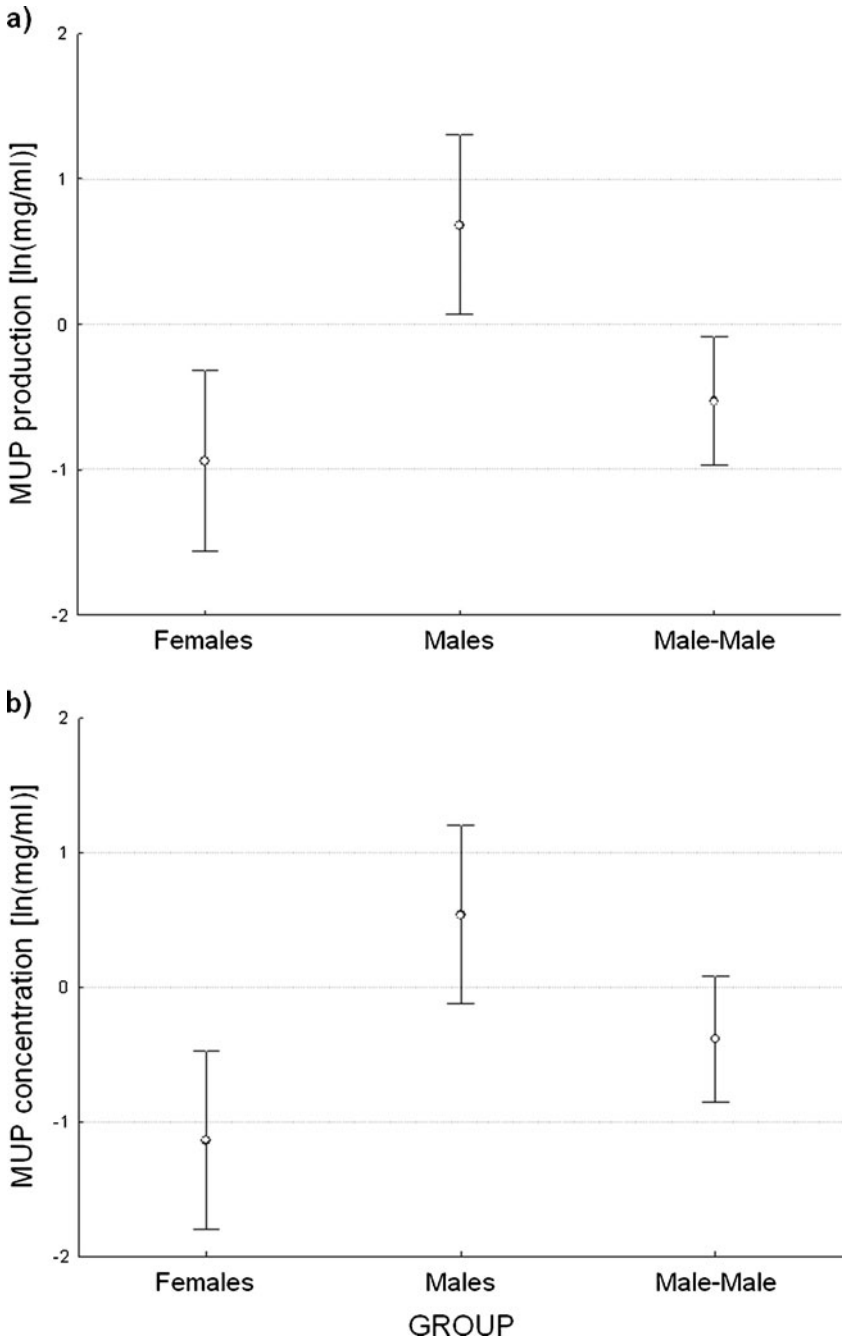


Fig. 2 Social modulation of major urinary protein (MUP) quantity in three groups of house mice, *Mus musculus musculus*: 1) females that were exposed to a male: Females, $N=10$; 2) males that were exposed to a female: Males, $N=10$; and 3) males that were exposed to another male: Male-Male, $N=10$. Values are expressed as a ratio of MUP quantity in social and individual experimental setup. The ratio was calculated by dividing the data obtained in the social experimental setup by the data from the individual experimental setup (S/I) due to big individual differences of MUP quantity. This ratio was transformed with the natural logarithm function. Dots represent means; bars represent 95% confidence intervals. Plot **a** demonstrates changes in MUP level (corrected with creatinine). The ratio S/I is in all three tested groups different from zero (confidence intervals do not overlap the zero line), which indicates that the animals respond significantly to the treatment. Positive values of the ratio S/I shows that Males up-regulated MUP level in social when compared to individual treatment while negative values obtained for the other two groups demonstrate that these animals down-regulated the production of MUPs. Plot **b** demonstrates change in MUP concentration (non-corrected with creatinine) where the only significant response gave Females (confidence intervals of the ratio S/I do not overlap zero line). Females lowered MUP concentration when exposed to males

Since the main aim of the study was to examine whether or not the fluctuation of MUP production due to social contact that is observed in laboratory mice, occurs and may play a role in chemical communication of wild-living mice, we used *M. m. musculus* trapped from different sites in the Czech Republic. Interestingly, not all animals in the study responded in the same way to social stimulation (see Results). Although it might be a result of random variation, factors may exist that were not fully controlled such as age, dietary conditions, or sexual experience, and that may be responsible for the distinct responses. This disadvantage that comes from using wild animals trapped as adults from their natural environment is, however, compensated by the fact that they experienced complex social conditions before housing in a laboratory, and thus, should display a full repertoire of social behavior.

The results show a high variability of MUP concentration among wild-living individuals that appears to be retained even after creatinine correction and, therefore, is not caused by changes in urine dilution but by different MUP production. Although sample size was rather low and the analysis thus has limited validity, it should be pointed out that we did not observe any difference of MUP variability among males from five different localities. Since

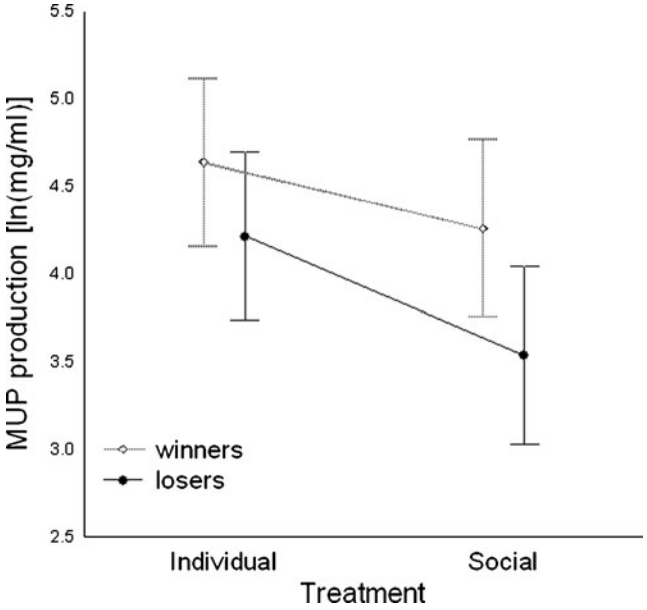


Fig. 3 Production of major urinary proteins (MUPs) in winner and loser male house mice, *Mus musculus musculus*. The data were transformed by using the natural logarithm function. Both male categories down-regulated the MUP level in social when compared to individual treatment. Repeated measures ANOVA revealed that the category of male (winner and loser) was also an important factor influencing males' response ($F_{(1,18)}$=4.67; P=0.044) showing that winners expressed more protein than losers $N_{(winner)}$=10; $N_{(loser)}$=10; bars represents 95% confidence intervals

there is still a lot of uncertainty about the mechanisms of the regulation of MUP production, it is difficult to suggest what genetic and regulatory factors underlie their individual quantitative variability and how they influence the properties of individuals' scent signals. Another interesting question is whether this characteristic of MUP production is specific for *M. m. musculus* or is common to other species and subspecies especially for *M. m. domesticus* in which the high qualitative variability has been demonstrated (Robertson et al., 1997).

Socially induced changes of MUP production that we observed may be mediated by common hormonal regulators. MUP expression is under the control of several hormones including testosterone (Wicks, 1941; Thung, 1956), the production of which is highly dependent on social conditions (Macrides et al., 1975; Van Oortmerssen et al., 1987; Oyegbile and Marler, 2005). Although from our experiments we are not able to define a direct regulatory agent responsible for the change of MUP expression level, testosterone is, in males, a probable candidate. Specifically, the up-regulation of MUP expression observed in males may be caused by an elevation of plasma testosterone that is known to be triggered by the introduction of a strange female to a male's homecage (Macrides et al., 1975). Interestingly, the scent marks of high testosterone males are more attractive for females than those of low testosterone males (Ferkin et al., 1994; Zala et al., 2004). This is presumably caused by increased production of male pheromones that act as female attractants (Jemiolo et al., 1985, 1991). Some of them are known to bind to MUPs (Bacchini et al., 1992; Novotny et al., 1999) and thus it is likely that up-regulation of MUPs contributes, directly or in coordination with the pheromonal ligands, to the enhancement of attractive properties of male chemical signals to females. Moreover, enhanced level of MUP expression may facilitate triggering ovulation in females, which has been demonstrated by Morè (2006).

Testosterone also may play a role in down-regulation of MUP production after male-male exposure which appeared

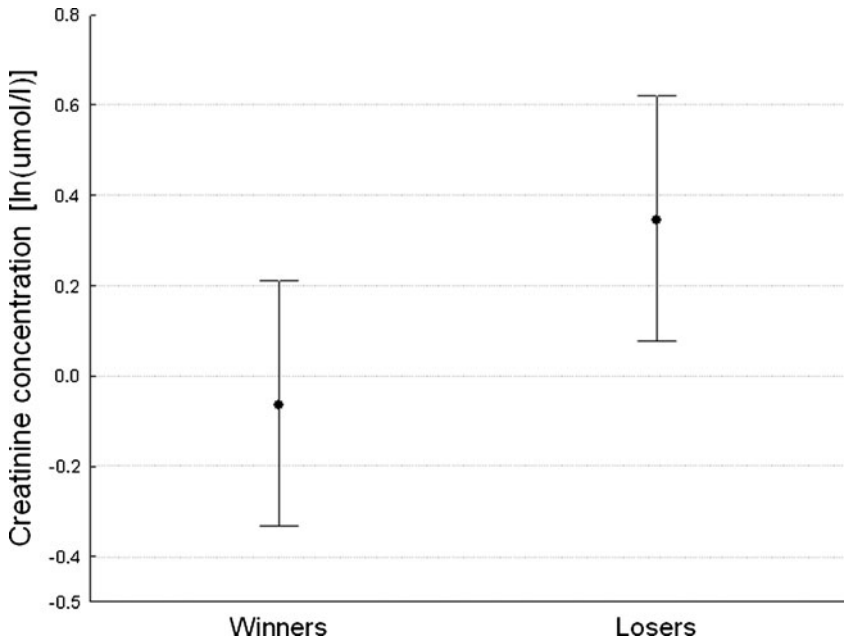


Fig. 4 Concentration of creatinine in winner and loser male house mice, *Mus musculus musculus*. Values are expressed as a ratio of creatinine quantity in social and individual experimental setup (S/I) and transformed with the natural logarithm function. Dots represent means; bars represent 95% confidence intervals. In social treatment losers significantly concentrated their urine which is demonstrated by the confidence intervals that do not overlap zero

to be more pronounced in those males that were defeated in intra-pair male-male encounters performed after the social part of experiment (Fig. 3) (Zielinski and Vandenbergh, 1993; Oyegbile and Marler, 2005). Moreover, in defeated males, an increased concentration of urinary creatinine was observed (Fig. 4), indicating that their urine was more concentrated than urine of winners. Interestingly, Drickamer (1995) reported a difference in urine production between dominant and subordinate males, with subordinates producing less urine than dominants. Although we did not measure the total excretion of urine, we suppose that higher concentration of urinary creatinine, which we observed in defeated males, might be caused by reduced urinary production. Since urine contains quite a few of aggression promoting molecules including MUPs (Novotny et al., 1990; Chamero et al., 2007), the lowering of its production and the reduction of overall scent marking in males of lower competitive abilities (Desjardins et al., 1973), reduces the probability of attack from a dominant male. In this context, it is noteworthy that the while concentration of creatinine in the urine of losers increased, concentration of MUPs remained unchanged due to down-regulation of their expression.

Surprisingly, female mice responded to male's presence with down-regulation of MUP expression and with reduction of MUP concentration in urine. This is in contradiction to the results of a previous study where C57BL/6 females up-regulated MUPs in the same experimental setup (Stopka et al., 2007). Since territory owners are known to be aggressive towards intruders of both sexes and thus a contact with a strange male represents a potentially dangerous situation for wild females (Rowe and Redfern, 1969; Hurst, 1990; Munclinger and Frynta, 2000), one possible explanation for our observation is that down-regulation of MUP expression is a consequence of stress response. Inbred mice C57Bl/6 that are genetically identical and have been demonstrated to show impaired ability to discriminate among members of the same laboratory strain (Nevison et al., 2003), might fail to show this response because they did not recognize a stimulus C57BL/6 male as a stranger (Nevison et al., 2000).

It is, however conceivable that quantitative changes of MUPs, molecules that are actively involved in mouse chemical communication, may have functional significance and influence responses of conspecifics to a female. For example, an earlier study suggested that inbred females may use MUPs to advertise their reproductive conditions to males (Stopka et al., 2007). However, in wild females exposed to a male presence, we did not find any evidence of fluctuation of MUP quantity in the course of the estrous cycle, which may indicate that the response was triggered more by unfamiliarity of a male than by the fact that it may be a potential mate. Therefore, more specific behavioral experiments have to be performed to elucidate a prospective role of MUPs in stabilization of social unit or in mate choice in wild house mice.

To reliably assess the quantity of proteins in urinary samples that may be diluted differently among individuals, the creatinine to protein ratio that corresponds to the (post) translational level of expression usually is calculated (Beynon and Hurst, 2003). However, in this study, we also analyzed data that were non-corrected with creatinine, thus reflecting an absolute concentration of MUPs in the urine. Considering these data, we were able to detect a difference in urine dilution between the winner and loser males. Our data support the work of Drickamer (1995), who showed that decreased production of urine is a mechanism that contributes to the maintenance of the characteristic pattern of urination in submissive males (Desjardins et al., 1973). The volume of urine produced influences the number of territorial marks that an individual can make, and the amount of protein deposited in the urine mark is dependent on both the production of protein and the volume of the mark. Therefore, the strength of the signal in the scent marks from two males with similar level of MUP expression but with different urine dilution may differ. For this reason, the concentration of MUPs should not be omitted from the analysis whenever the quantity of MUPs is being assessed.

We elected to use a densitometric analysis that permitted us to measure the amount of a particular protein in a mixture instead of the Bradford protein assay (Bradford, 1976), which determines the total concentration of all proteins in a sample. As standard for the densitometric analysis, we chose carbonic anhydrase (molecular weight of 29 kDa), which is only slightly higher than that of MUPs.. Carbonic anhydrase contains a comparable amount of dye binding sites to MUPs, in contrast to the frequently used bovine serum albumin standard (60 kDa). The use of the latter standard may result in underestimation of the MUP concentration.

The results of this study further our knowledge of MUPs in that it shows a plasticity of their expression in different social contexts. This plasticity is supposedly mediated by the hormonal regulation, primarily by testosterone. In wild females, however, the regulatory role of testosterone and other hormonal agents remains uncertain. Studies that have examined hormonal regulation of MUPs to date, are, unfortunately of limited value because they usually tested laboratory or even hormone-deficient mice and treated them with unnaturally high hormone levels (Knopf et al., 1983; Johnson et al., 1995). Therefore, to get better insight into the roles of MUPs, there is need to characterize ultimate as well as proximate factors that modulate MUP expression in wild animals under more natural conditions.

Another significant result of this study shows that expression change of MUPs in males depend on the sex

of the animal introduced into a male's cage. The up-regulation that was observed in response to females indicates that for males, MUPs may be more important in communication between, rather than within, sexes. Given the overall cost of scent-marking and production of high amounts of protein and volatile pheromones, it can be hypothesized that scent marks are honest displays of males' quality (Penn and Potts, 1998; Gosling and Roberts, 2001). MUPs may, therefore, play a role in the process of mate choice by increasing the attractive properties of a male's scent marks and advertising his fitness to females.

Our study was performed on the Eastern European subspecies of the house mouse (*M. m. musculus*), which differs slightly in several aspects from *M. m. domesticus*. These include behavioral (Christophe and Baudoin, 1998; Smadja and Ganem, 2002), morphological (Boursot et al., 1993) differences as well as the specific level of MUP expression (Stopkova et al., 2007). Whether they differ also in the function of MUPs in chemical communication is unclear (Janotova and Stopka, 2009). Therefore, we emphasize that further studies in *M. m. domesticus* are needed to determine to what extent our findings are applicable to this subspecies.

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Identification of Camphor Oxidation and Reduction Products in *Pseudomonas putida*: New Activity of the Cytochrome $P450_{cam}$ System

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Abstract P450 enzymes are known for catalyzing hydroxylation reactions of non-activated C-H bonds. For example, $P450_{cam}$ from *Pseudomonas putida* oxidizes (1*R*)-(+)-camphor to 5-*exo*-hydroxy camphor and further to 5-ketocamphor. This hydroxylation reaction proceeds via a catalytic cycle in which the reduction of dioxygen (O_2) is coupled to the oxidation of the substrate. We have observed that under conditions of low oxygen, *P. putida* and isolated $P450_{cam}$ reduce camphor to borneol. We characterized the formation of borneol under conditions of low oxygen or when the catalytic cycle is shunted by artificial oxidants like *m*-chloro perbenzoic acid, cumene hydroperoxide, etc. We also tested the toxicity of camphor and borneol with *P. putida* and *Escherichia coli*. We have found that in *P. putida* borneol is less toxic than camphor, whereas in *E. coli* borneol is more toxic than camphor. We discuss a potental ecological advantage of the camphor reduction reaction for *P. putida*.

Key Words Aerated and non-aerated cultures · Borneol · Gas chromatography-mass spectrometry (GC-MS) · Nuclear magnetic resonance spectroscopy (NMR) · P450 · Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) · Reduction reaction · Toxicity assays

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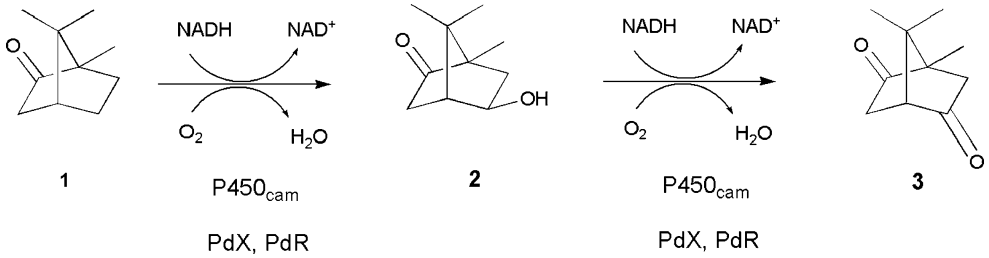
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Introduction

Cytochrome $P450_{cam}$ (CYP101) is a heme monoxygenase (Sligar et al., 1974; Sono et al., 1996) isolated from the soil bacterium *Pseudomonas putida* (Davydov et al., 2001), which utilizes camphor as a carbon source. $P450_{cam}$ is known to catalyze the hydroxylation of camphor (**1**) to 5-*exo*-hydroxycamphor (**2**) (Sibbesen et al., 1996; Altun et al., 2007) and further to 5-ketocamphor (**3**) (Auclair et al., 2001). $P450_{cam}$ needs two electron transfer proteins, putidaredoxin (PdX) and putidaredoxin reductase (PdR) (Katagiri et al., 1968) for its catalytic activity (Lipscomb et al., 1976; Pochapsky et al., 2003). The catalytic cycle of the enzyme has evolved to couple the highly favorable reduction of O_2 to the unfavorable oxidation of the hydrocarbon substrate. A key intermediate in the catalytic cycle, the iron-oxo species (Fe(IV)=O), is supposed to be the most reactive species (Makris et al., 2006), and also is believed to form via a shunt pathway (Schunemann et al., 2004; Newcomb et al., 2006) when artificial oxidants such as iodosobenzene, cumene hydro-peroxide (Chefson and Auclair, 2006), or *meta*-chloro perbenzoic acid (*m*-CPBA) are used (Egawa et al., 1994).

Some *P. putida* contain a plasmid (the CAM plasmid) that encodes a degradation pathway for the monoterpene (1*R*)-(+)-camphor (Aramaki et al., 1993). The first step in that pathway is the oxidation of camphor at position 5 (Gould et al., 1981). This step is catalyzed by the $P450_{cam}$ complex with its redox partners PdR and PdX (Gunsalus and Sligar, 1976; Shimada et al., 2001). We identified 5-*exo*-hydroxycamphor (**2**) and 5-ketocamphor (**3**) (Scheme 1) in enzymatic assays performed with the purified proteins isolated from *P. putida* (Rojubally, 2007).

Here, we describe the identification of a third product borneol (**4**) from assays involving the peroxide shunt and



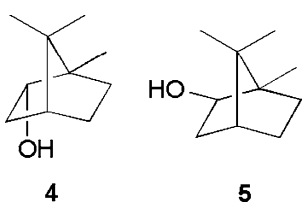
Scheme 1 The hydroxylation reaction of camphor. formation of 5-*exo*-hydroxy camphor and 5-Ketocamphor

for assays conducted under regular catalytic conditions. This finding is surprising, because borneol, a reduction product of camphor, is formed under conditions that are not considered to be reducing. Also surprisingly, isoborneol (**5**) was not detected (Scheme 2). We have found that cytochrome $P450_{cam}$ produces borneol, and we describe the conditions under which it forms *in vivo* and *in vitro*. We also compared the toxicity of camphor and borneol against strain ATCC 17453 of *P. putida* (containing the cam plasmid) and against *Escherichia coli*, a bacterium that lacks cytochrome P450 (Cryle et al., 2003). Our results suggest that the formation of borneol from camphor may confer an advantage to *P. putida*.

Methods and Materials

General Methods and Chemicals Experimental procedures and full spectroscopic details are available for all compounds in the Supplementary Data section. All solvents were distilled prior to use. Reduced nicotine adenine dinucleotide (NADH), dithiothreitol (DTT), lysozyme, DNase, RNase, Vitamin B_1, riboflavin, δ-aminolevulinic acid, hydrogen peroxide (used for assays), protease inhibitors leupeptin, aprotinin, and 4-(2-aminoethyl)-benzenesulfonyl fluoride were purchased from Sigma-Aldrich (Oakville, ON, Canada); dimethyl sulfoxide (DMSO, 99.9% chemical purity) from Caledon Laboratories Ltd (Georgetown, ON, Canada). Reagent grade 1-indanone was purchased from Eastman Co. (Kingsport, TN, USA) and used without further purification. *m*-Chloro perbenzoic acid (≤77% c.p.) was purchased from Sigma-Aldrich; purified by known methods (Aggarwal et al., 1998), and used for assays. Other shunting agents, cumene hydro-peroxide (CHP), sodium periodate ($NaIO_4$), and sodium hypochlorite (bleach) were used as received. Gas chromatography was performed on a Hewlett Packard 5890 GC (Avondale, PA, USA) equipped with a flame ionization detector and a 30 m×0.25 mm i.d.×0.25 μm film thickness SPB-5 column (Supelco, Bellefonte, PA, USA). The instrument was programmed as follows: 80°C (5 min), 10°C/min to 200°C (4 min), 50°C/min to 250°C (20 min); head pressure 15 psi; total flow through the column was 1.7 ml/min with injector and detector temperatures set at 250°C. Enantiomers of borneol and isoborneol were separated on a 30 m×0.25 mm i.d.×0.25 μm film thickness CycloSil B column (J & W Scientific, Folsom, CA, USA), with the column oven programmed isothermally at 120°C and a head pressure 15 psi. Gas chromatography-mass spectrometry (GC-MS) was carried out on a Varian Saturn 2000 MS (Mississauga, ON, Canada) equipped with the identical SPB-5 column (Supelco), and the column was programmed as follows: 45°C (0.5 min), 7°C/min to 120°C (1 min), 50°C/min to 260°C (3 min) with injector and transfer line temperatures set at 220°C and 260°C, respectively. Electron impact spectra were obtained at an emission current of 30 μA, scanning from 50 to 365 amu, with ion storage (SIS mode) 49–375, trap temperature 170°C and transfer line 250°C. For sensitivity measurements, electron impact spectra were obtained with ion storage (SIS mode) 90–170.

UV/Vis spectra were obtained on a Cary 300 Bio UV-visible double beam instrument (Mississauga, ON, Canada). NADH utilization rates and hydrogen peroxide formation were measured on a thermostatted Hach DR/4000 U spectrophotometer (Mississauga, ON, Canada). The dissolved oxygen in the aerated and poorly aerated cultures was determined by an Orion DO meter (model 850) (Beverly, MA, USA). Activity assays were carried out at 22°C. Electrophoresis was performed on polyacrylamide gels (14%, 29:1) with 0.5% sodium dodecyl sulfate (SDS-PAGE). Samples were reduced by treatment with DTT (31 mg/ml) before loading on gels. Gels were stained with Coomasie Brilliant Blue R (Sigma). Samples were sonicated in a Branson Ultrasonic sonicator (Danbury, CT, USA). Centrifugations were carried out with a Beckmann Avanti J-26 XPI centrifuge (Mississauga, ON, Canada), equipped with a JLA 8.1000 rotor.



Scheme 2 Borneol (**4**), one possible reduction product of camphor, and isoborneol (**5**). borneol is formed in *P.Putida* but not isoborneol

The buffers used were: lysis (20 mM phosphate buffer (K^+), pH 7.4 with 1 mM camphor; T-100 (50 mM Tris, 100 mM KCl, pH 7.4); and T-400 (50 mM Tris, 400 mM KCl, pH 7.4). Substrate-free P450 was prepared by passing the substrate-bound enzyme over a Sephadex G-10 column equilibrated with 100 mM MOPS (pH 7.0) (Gunsalus and Wagner, 1978). The concentration of the substrate-free P450 was calculated by the ε_{417}=104 mM^{-1} cm^{-1}. GC-MS analysis of the chloroform extract of the substrate-free P450 confirmed that less than 2% of the camphor was bound to P450.

(1R)-(+)-Camphor Purification (1*R*)-(+)-Camphor (Sigma-Aldrich) contained ~5% borneol as confirmed by GC-MS in comparison with authentic borneol (Sigma). The camphor was recrystallized from ethanol/water and then purified on silica gel with a hexane:ethyl acetate gradient [from 1:0 (30 fractions, 8 ml) to 99:1 (5 fractions), to 4:1 (8 fractions)]. The (1*R*)-(+)-camphor was chemically pure (GC-MS). Mass spectrum (EI): *m/z* (% of base peak) 153 (M+1, 6), 152 ($M^{\cdot+}$, 8), 137(7), 108 (66), 95 (100), 69 (15), 67 (55), and 55 (28) and CI (isobutene): *m/z* (% of base peak) 153 (M+1, 100), 137 (2), 135 (6), 108 (10), 95 (3), 81 (10), 69 (12), and 67 (20). Other products, 5-ketocamphor and 5-*exo*-hydroxy camphor were synthesized by known methods (Dauben et al., 1956; Darby et al., 1979) and were characterized by nuclear magnetic resonance spectroscopy (NMR) and GC-MS.

Nuclear Magnetic Resonance Spectroscopy (NMR) The proton and the carbon nuclear magnetic resonance spectra were run on a Varian 500 MHz instrument (Varian Inc., Palo Alto, CA). The chemical shifts (δ) for all compounds are listed in parts per million with NMR solvent ($CDCl_3$) as an internal reference. The coupling constants (*J*) are listed in Hertz (Hz).

Protein Production (from P. putida) and the Purification Steps $P450_{cam}$, PdR, and PdX were isolated from *P. putida* (ATCC 17453) according to the protocol reported by (Gunsalus and Wagner, 1978). *Pseudomonas putida* were grown in nutrient broth (Gibco) containing 0.5 mg/ml of (1*R*)-(+)-camphor with shaking (250 rpm) to A_{600}=0.9–1.0. Cells from 2 l of culture were lysed in 50 ml of 20 mM potassium phosphate, pH 7.4, 1 mM camphor. Cells were suspended in 10 mM in EDTA (pH 7.4). Then, lysozyme (100 mg/l) and camphor (1 mM in ethanol) were added and stirred for 30 min at 4°C. The suspension was sonicated with 50% duty cycle for 10 min and brought to 10 mM in $MgSO_4$. DNase (2 mg, Sigma) and RNase (10 mg, Sigma) were added and stirred for 30 min at 4°C. The lysate was sonicated for 5 min and homogenized in a tissue homogenizer with a Teflon pestle. The homogenized cells were harvested by centrifugation (7000×*g*, 30 min), and dialyzed with frequent changes of 20 mM potassium phosphate (pH 7.4) containing 1 mM camphor for further purification by ammonium sulfate precipitation. The dialyzed lysate of *P. putida* was subjected to a 20% ammonium sulfate cut to remove the cell debris. The 20% supernatant was carried forward to 55% ammonium sulfate saturation to isolate the proteins. All three proteins were isolated together in the above step. Proteins were further purified by column chromatography (DE-52 column with a linear gradient of buffer T-100 to T-400 containing 1 mM camphor and 1 mM β-mercaptoethanol at 1 ml/min). Fractions with high absorbances at A_{392} (in the case of P450), A_{454} (in the case of PdR), and A_{325} (in the case of PdX) were pooled, and checked by SDS-PAGE before proceeding with gel-filtration chromatography. The collected fractions were concentrated in an Amicon ultrafiltration cell equipped with a 10 kDa membrane (Millipore Corporation, Billerica, MA, USA), and the concentrate (containing P450, PdR, and PdX) was loaded onto a S-100 column, eluted with T-100 buffer, 1 mM sucrose, and 1 mM camphor at 1 ml/min. The three proteins could not be separated, and the selected fractions containing A_{280}/A_{392}<7.0, A_{280}/A_{454}<9, A_{280}/A_{325}<4.0 were analyzed by SDS-PAGE for molecular weight determination and taken for assays.

In vivo Assays with P. putida To monitor product formation under aerated and non-aerated conditions, *in vivo* assays were performed with *P. putida* cultures. Culture assays were performed in 500 ml Erlenmeyer flasks. A dense culture (A_{590}=0.9) was treated with 100 mg of camphor delivered in dimethyl sulfoxide (DMSO). Cultures were monitored by removing 1 ml of culture, adding 0.2 ml of saturated NaCl solution, and extracting with 1 ml of chloroform. The organic layer was dried over Na_2SO_4 and analyzed by GC-MS. Aerated conditions (dissolved oxygen was measured to be 11 mg/l) were obtained in small flasks (250 ml) by shaking at ~250 rpm, whereas poorly aerated conditions (dissolved oxygen was measured to be 2 mg/l) were obtained in 10 ml tubes with very slow shaking (50 rpm).

To monitor the conversion of camphor to metabolites by the induction of P450 under aerated and non-aerated conditions, *P. putida* ATCC 17453 strain was grown for seven generations in two replicates without camphor. Aliquots (20 μl) of the 7th generation cultures (both aerated and non-aerated) were inoculated into 100 ml of nutrient broth medium in a 250 ml flask. This 8th generation of log phase culture was treated with 0.77 g of camphor delivered in DMSO, and the absorbances at 280, 392, and 410 nM were recorded at 0 min (immediately after adding camphor), 5, 30, 60, 120, 180, 240 min, and 8 h under both aerated and non-aerated conditions. At every time point, the sample was centrifuged to collect the

pellet and the supernatant. Supernatants from the different time points were extracted with $CHCl_3$/indanone (7.2 μM) for the extraction of the product(s) and the substrate. Indanone was our internal standard for the quantification of products by GC-MS. A second extraction of the aqueous phase with 0.7 ml of $CHCl_3$/indanone followed. The combined organic layers were dried over $MgSO_4$ and analyzed by GC-MS.

In vitro Assays with Isolated $P450_{cam}$, PdR, and PdX Complex In vitro enzymatic assays were performed in 2 ml of 50 mM phosphate buffer (100 mM K^+) (pH 7.4). The buffer was sparged with charcoal filtered air/oxygen or argon (both Sigma-Aldrich) prior to use. Concentrations of ferric P450 (with camphor), PdR, and PdX were determined by their extinction coefficients ε_{392}=104 mM^{-1} cm^{-1}, ε_{454}= 10.0 mM^{-1} cm^{-1}, and ε_{325}=15.6 mM^{-1} cm^{-1} (Gunsalus and Wagner, 1978). The reaction mixture contained 1.5 μM $P450_{cam}$, 2.5 μM PdR, 5 μM PdX, and the substrate camphor (2.5 mM). NADH consumption was initiated by the addition of 2.5 μl NADH stock (200 mM) and monitored for 10 min at 340 nm. The experiment was performed in four replicates, and three controls were run in parallel with the treatments: 1) blank: without enzyme but all other reactants added; 2) enzyme and the substrate added to the buffer without NADH; and 3) NADH and the enzyme added without camphor. The reaction was stopped by the addition of 1 ml $CHCl_3$/indanone (7.2 μM) for the extraction of the product(s) and the substrate. Indanone was our internal standard to quantify products by GC-MS. A second extraction of the aqueous phase with 0.7 ml of $CHCl_3$/ indanone followed. The combined organic layers were dried over $MgSO_4$ and analyzed by GC-MS.

In assays involving *m*-CPBA as a shunt agent for the catalytic cycle, the reaction mixture contained 1.5 μM $P450_{cam}$, *m*-CPBA (1 mM), and substrate camphor (1 mM). The experiment was performed in four replicates, and three controls were run in parallel with the treatments: 1) blank: without enzyme but all other reactants added; 2) enzyme and the substrate added to the buffer without *m*-CPBA; and 3) *m*-CPBA and the enzyme added without camphor. The reaction mixture was incubated for 15 min at 22°C and extracted in the same way as above. The dried organic layers were analyzed by GC-MS.

In assays involving borneol as the substrate and *m*-CPBA as a shunt agent for the catalytic cycle, the reaction mixture contained 2 μM $P450_{cam}$, *m*-CPBA (1 mM), and substrate (1 mM). The experiment was performed in four replicates, and three controls were run in parallel with the treatments: 1) blank: without enzyme but all other reactants added; 2) enzyme and the substrate added to the buffer without *m*-CPBA; and 3) *m*-CPBA and the enzyme added without borneol. The reaction mixture was incubated for 30 min at 22°C and extracted in the same way as above. The dried organic layers were analyzed by GC-MS.

Toxicity Assays of Tetracycline, DMSO, Camphor, and Borneol against P. putida and E. coli Cultures of the strains ATCC 17453 of *P. putida* and XL-1-blue of *E. coli* were grown overnight, and used to inoculate 2×250 ml growing cultures, which were grown at 300 rpm with good aeration until an O.D. of 0.1 was reached. An aliquot (5 ml) of each of these cultures was transferred separately to Falcon tubes in three replicates, to each of which either camphor, borneol, or tetracycline was added to a final concentration of 1 mM. Dimethyl sulfoxide (DMSO), the solvent used for preparing stocks, also was used as one of the controls. Cultures were grown for 1 hr and incubated overnight at 4°C. They were diluted 10^3×, 10^4×, 10^5×, 10^6×, 10^8× in three replicates and then plated on non-antibiotic LB-agar plates in two replicates (20 and 50 μl) and were incubated overnight at 27°C. Colonies were counted after overnight incubation. The toxicities of camphor and borneol to the two strains were assessed by measuring the reduction of cell density [colony forming units (CFU)/ml]. To obtain CFU/ml, colonies were grown on agar plates and the numbers of colonies were divided by the volume plated. This, when divided by the dilution factor, gives the CFU/ml in the original sample. We compared *P. putida* with the Cam plasmid to *E. coli*, a species of bacterium known to not contain any P450 genes (Cryle et al., 2003). A comparison of the relative toxicity between borneol and camphor on *E. coli* was determined by a one-way ANOVA test (Vander Waerden test) with JMP starter software (Cary, NC, USA) with significance determined at α=0.05. The distribution of the data was checked with JMP software, and the normal hypothesis was rejected. Therefore, a non-parametric method was used for the analysis. Tukey-Kramer pairwise comparisons were performed to test the effects of camphor and borneol relative to DMSO; Wilcoxon and Vander Waerden comparisons were used to test the effects of camphor and borneol directly.

IC_{50} Experiments with Camphor and Borneol Against E. coli Two growing cultures (250 ml each) were inoculated with an overnight grown culture of XL-1-blue of *E. coli* and then grown at 300 rpm with good aeration until a O.D. of 0.1 was reached. An aliquot (5 ml) of this culture was transferred to Falcon tubes to which either borneol or camphor was added in varying concentrations. The final concentrations of these compounds ranged from 0.5 nM to 1 μM. Dimethyl sulfoxide (DMSO), the solvent used for preparing stocks, also was used as one of the controls. Cultures were grown for 1 hr and incubated overnight at 4°C. They were diluted 10^4× and 10^5× in three replicates and then plated on non-antibiotic LB-agar plates in two replicates (20 and 50 μl) and

incubated overnight at 37°C. The concentration of colony forming units (CFU/ml) was calculated the next day, depending on the number of colonies. The IC_{50} was calculated by graphing CFU/ml vs. the logarithm of concentration of compounds (Vrzal et al., 2010).

Synthesis of Standards The synthesis of 5-*exo*-hydroxycamphor (**2**) was initiated from commercially available borneol (**4**) or from racemic isoborneol (**5**) (Scheme 2). Either borneol was transformed into its acetate (**6**), and oxidation with chromium trioxide in acetic anhydride and acetic acid afforded the intermediates (**8**) or (**11**). The oxidation of compounds **8** and **11**, with pyridinium chlorochromate (PCC), afforded 5-ketocamphor (**3**). The selective reduction of 5-ketocamphor (**3**) with sodium borohydride yielded 5-*exo*-hydroxycamphor (**2**). 5-*exo*-Hydroxyisoborneol (**13**) was obtained as a major product from the complete reduction of 5-ketocamphor (**3**) or (**11**), and 5-*exo*-hydroxyborneol (**12**) was obtained by the reduction of (**8**) (Scheme 3). To monitor the bioconversion of camphor *in vivo* and *in vitro* by *P. putida* enzymes, deuterated camphor (3-D_1-camphor) **14** was synthesized (Scheme 4) from camphor **1** with D_2O in the presence of pyridine. The method for complete syntheses of all these compounds with its characterization is provided in the Supplementary Data.

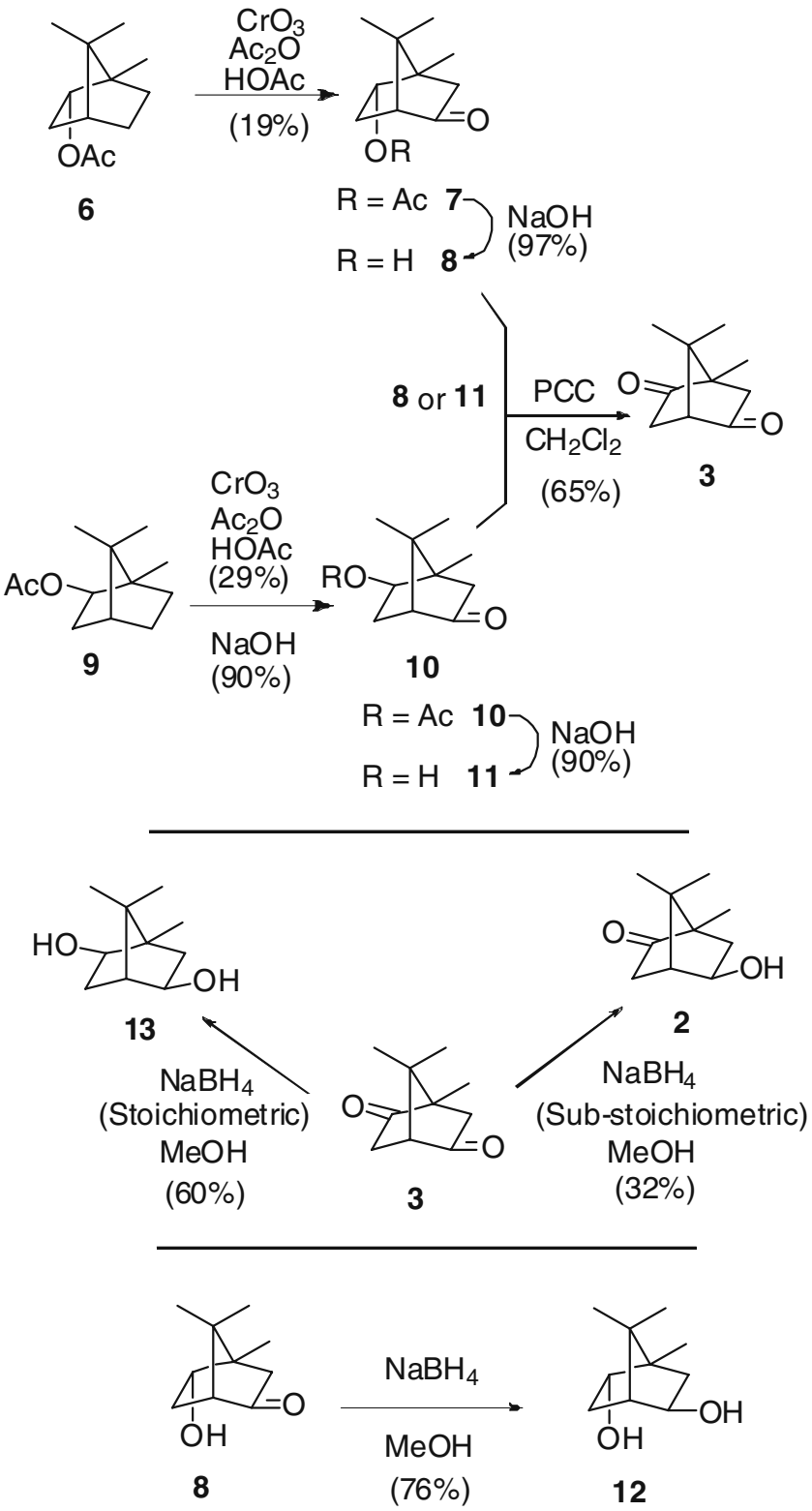
Results

Analysis of Products Formed in Pseudomonas putida Culture with (1R)-(+)-Camphor During in vivo Assays with Purified $P450_{cam}$ System *In vivo* production of the metabolites from an induced *P. putida* culture was monitored by extracting samples with chloroform and analyzing them by GC-MS. Under aerated conditions, 5-*exo*-hydroxycamphor (**2**) and 5-ketocamphor (**3**) were obtained as reported by Gould et al. (1981). In addition to **2** and **3**, 5-*exo* hydroxyborneol (**12**) and borneol (**4**) were detected (Fig. 1). In the poorly aerated system, borneol (**4**) was detected in the early stage of the reaction, its quantity gradually peaked around 4 hr, and then decreased, whereas 5-*exo*-hydroxyborneol (**12**), the other reduction product, formed at a later stage in the metabolic pathway (Fig. 2a). 5-Ketocamphor (**3**) formed only after 5 hr of incubation, whereas 5-*exo*-hydroxy camphor (**2**) was not detected in the poorly aerated system (Fig. 2a). In the non-aerated system, the quantity of borneol (**4**) increased steadily and did not appear to be bioconverted any further. The oxidation products, 5-*exo*-hydroxycamphor (**2**) and 5-ketocamphor (**3**), were present at low levels in the non-aerated system (Fig. 2b).

In vivo production of borneol and 5-ketocamphor was monitored under aerated and non-aerated conditions in growing *P. putida* cultures that had been treated with camphor. The quantities of borneol under both aerated and non-aerated conditions increased in the first 3 hr and then reached a plateau (Fig. 3). To prove that these products were obtained from camphor, deuterated camphor (3-D_1-camphor) **14** was synthesized (Scheme 4) from camphor **1**. (refer to Supplemental Data for complete synthesis). When 3-D_1-camphor **14** was added to a growing culture of *P. putida* and under oxidizing conditions, deuterated 5-ketocamphor was detected. Borneol detected under poorly aerated conditions was identified by chiral stationary phase GC-FID with a retention time of 29.8 min and compared to the retention times of two products of sodium borohydride reduced (1*R*)-(+)-camphor, borneol (**4**), and isoborneol (**5**). Isoborneol (**5**), eluted from the cycloSyl B column with a retention time of 25.8 min. This confirms that borneol is the only reduction product detected in the *P. putida* culture (Rojubally, 2007).

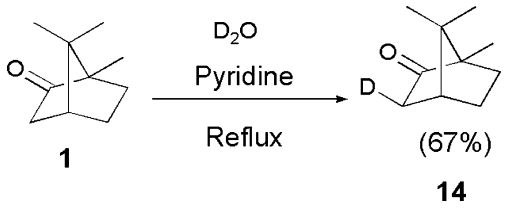
In vitro Assays with the Isolated $P450_{cam}$, PdR, and PdX Complex To determine whether $P450_{cam}$ is involved in the formation of **2**, **3**, and **4**, experiments were performed with purified, isolated $P450_{cam}$ and its redox partners, obtained from *P. putida* induced with camphor. The first unexpected result was that these proteins cannot be separated during the purification process but instead form a ternary complex. We, therefore, used this ternary complex in our *in vitro* assays.

Under oxygenated conditions, (when the assay buffer was sparged with oxygen or air), the enzyme complex catalyzed the oxidation of camphor to 5-ketocamphor (as the major product) and reduction to borneol (minor product) (Table 1). When there was insufficient oxygen for the catalysis (when the assay buffer was sparged with argon), surprisingly, borneol formed and 5-ketocamphor was not detected. Under these conditions, when the enzymatic pathway was shunted in the presence of *m*-CPBA and cofactor NADH was added, the amount of 5-ketocamphor formed was the same as that of borneol. In this assay, the concentration of 5-ketocamphor formed was less when compared to the oxygenated conditions. When hydrogen peroxide was used in place of *m*-CPBA under similar conditions, 5-ketocamphor was not detected and less borneol was formed (Table 1). In the absence of cofactor in the above mentioned conditions and when *m*-CPBA was used as a shunt agent, 5-ketocamphor was not detected and surprisingly more borneol was formed than with H_2O_2. This illustrates that the cofactor NADH is not responsible for the borneol formation. Further evidence that NADH is not the source of electrons was obtained when NADD was added to the assay reaction mixture that contained the substrate camphor and the enzyme, and



Scheme 3 Synthesis of 5-*exo*-hydroxy camphor (**2**), 5-ketocamphor (**3**) and 5-exo-hydroxy borneol standards

deuterated borneol was not detected under GC-MS in comparison with its synthetic standard (Rojubally, 2007) (Data not shown). Hydrogen peroxide, when added to the system, appears to be inhibitory, giving lower amounts of borneol. Taken together, these observations suggest that borneol forms under conditions when the bacteria begin to be stressed by low oxygen levels, but are still metabolizing camphor.



Scheme 4 Formation of 3-D_1-camphor (**14**) from camphor (**1**)

Enzymatic Assays Involving Borneol as the Substrate The enzymatic assays involving borneol as the substrate were conducted under shunt conditions and the products formed were monitored by GC-MS. We demonstrated the conversion of borneol to camphor and further to 5-*exo*-hydroxycamphor, which shows that this formation of borneol is a reversible reaction. The concentration of camphor was 33± 1.6 nmol/min/μmol P450, and 5-*exo*-hydroxycamphor was 50±1.6 nmol/min/μmol P450. This illustrates that *P. putida* can convert borneol back to camphor.

Toxicity Assays for Camphor and Borneol To test for a potential adaptive value for the conversion of camphor to borneol, we compared the relative toxicities of camphor, borneol, and tetracycline on *P. putida* and on *E. coli*. The ANOVA on mean cell densities for each species revealed an effect of treatment (*P. putida*: P=0.006 and F=4.40; *E. coli*: P< 0.001 and F=8.63). Tukey-Kramer pairwise comparisons of the toxicity of the substrates to *P. putida* revealed no significant differences in cell densities following monoterpene treatment relative to DMSO, suggesting that neither camphor nor borneol were toxic to *P. putida* (Fig. 4a) (DMSO/camphor pair, P=0.393; DMSO/borneol pair, P=0.981; and camphor/borneol pair, P=0.629). In contrast, treatment with tetracycline did cause a reduction in cell density relative to DMSO (Fig. 4a) (DMSO/

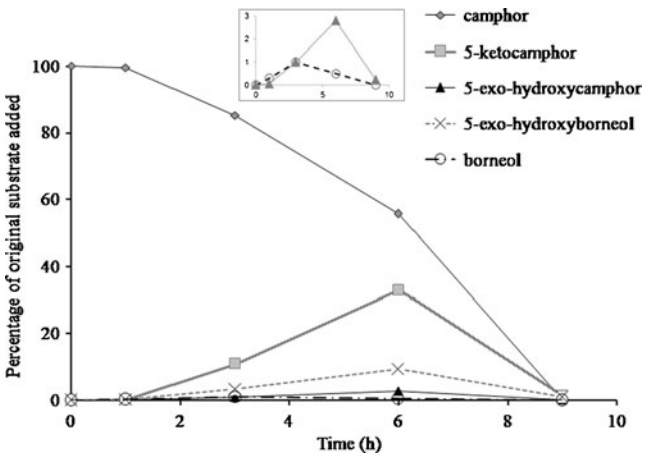


Fig. 1 Time course of relative quantities of metabolites measured from an induced culture of *Pseudomonas putida* under aerated conditions. The figure in the inset shows the time course for 5-*exo*-hydroxy camphor and borneol

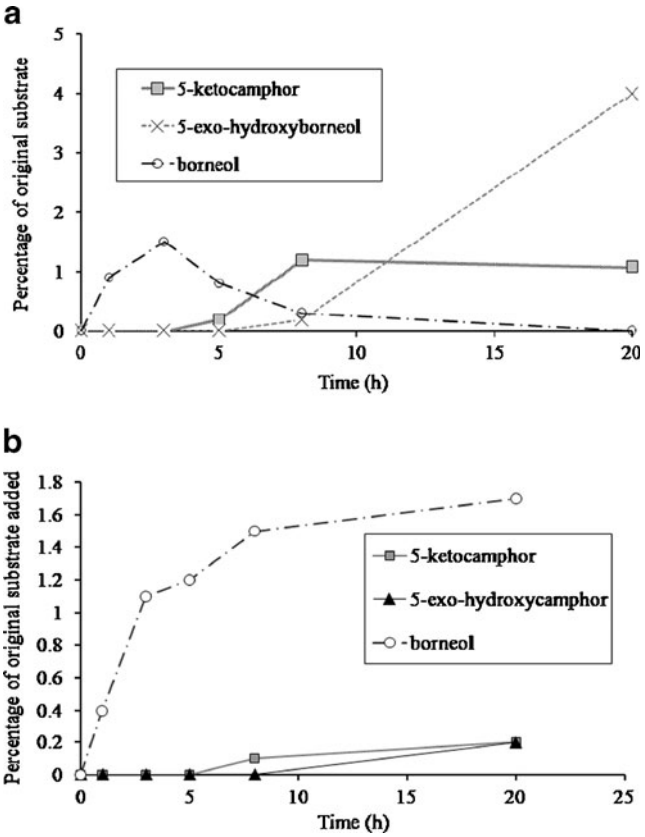


Fig. 2 Time course of relative quantities of metabolites measured from an induced culture of *Pseudomonas putida* under poorly-aerated (**a**) and non-aerated (**b**) conditions

tetracycline pair, P=0.007; camphor/tetracycline pair, P= 0.320; and borneol/tetracycline pair, P=0.022). Wilcoxon and Vander Waerden tests revealed that camphor was more toxic than borneol to *P. putida* (Fig. 4a) (P=0.025, N=3). Tukey-Kramer pairwise comparisons of the toxicity of the substrates to *E. coli* revealed that camphor, borneol, and tetracycline were all toxic to *E. coli* relative to DMSO (Fig. 4b) (DMSO/camphor pair, P<0.001; DMSO/borneol pair, P<0.001; DMSO/tetracycline pair, P<0.001; camphor/borneol pair, P=1.0; camphor/tetracycline pair, P=1.0; and borneol/tetracycline pair, P=1.0). Wilcoxon and Vander Waerden comparisons revealed that while both monoterpenes were toxic to *E. coli*, borneol was more toxic than camphor (P=0.035, N=3). In addition to this, the IC_{50} of borneol with growing cultures of *E. coli* ranged from 14.3 to 20 nM, whereas the IC_{50} of camphor ranged from 19 to 42 nM (Fig. 5).

Discussion

Hydroxylation of camphor is catalyzed by the $P450_{cam}$ complex with its redox partners PdR and PdX (Gunsalus

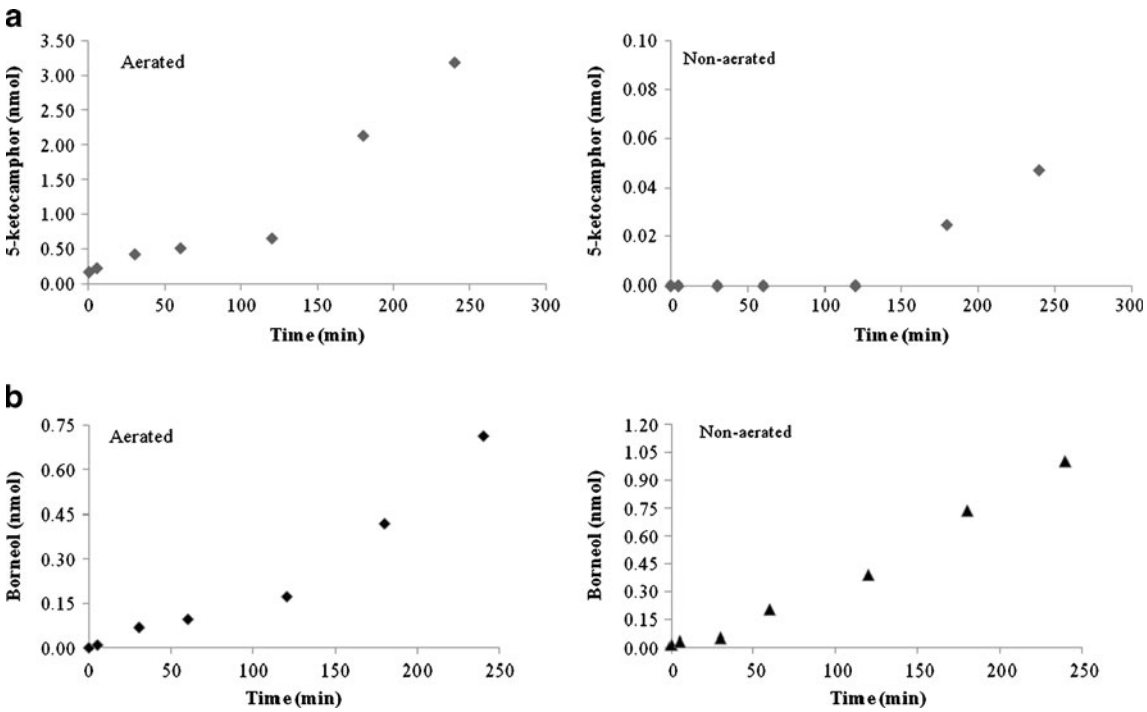


Fig. 3 Time course of quantities of 5-ketocamphor (**a**) and borneol (**b**) from an induced culture of *Pseudomonas putida* under aerated and non-aerated conditions

and Sligar, 1976; Shimada et al., 2001). The P450 reduces O_2 with electrons from NADH and incorporates one oxygen atom into product (Guengerich and MacDonald, 1990). Here, we observed that, in addition to the known camphor oxidation products 5-*exo*-hydroxycamphor (**2**) and 5-ketocamphor (**3**), a reduction product, borneol (**4**),

Table 1 Assays with ternary P450-PdR-PdX complex isolated from *Pseudomonas putida*[a]

Enzymatic assay	Products[b]		NADH consumed[c]	H_2O_2 formed[d]
	Borneol (**4**)	5-ketocamphor (**3**)		
O_2[e]	2.4±2	207±27	763±181	0
Air[f]	65±7	484±121	1303±484	13.6±9
Argon[g]	20±8	0	0	15.9±8
Argon + *m*-CPBA + NADH[h]	24±8	32±4	3±1	ND[i]
O_2[e]	0	272±36	606±10	0
Argon + *m*-CPBA[j]	71±48	0	NA[i]	ND[i]
Argon + NADH + H_2O_2[k]	7±5	0	ND[i]	ND[i]

[a] Table entries are mean ± s.e. (N=4); substrate (camphor) concentration: 2.5 mM for the assays

[b] Products detected are reported in nmol/min/μmoles P450

[c] NADH consumed is reported in nmol/min/μmoles P450

[d] Hydrogen peroxide formed (2 electron uncoupling) reported in nmol min/μmoles P450

[e] Oxygen was sparged into the buffer for enzymatic assays

[f] Charcoal filtered air was sparged into the buffer for enzymatic assays

[g] The buffer was sparged with argon for the assays

[h] The buffer was sparged with argon in the shunt pathway (*m*-CPBA) and the cofactor was added

[i] *NA* not applicable, *ND* not determined

[j] The buffer was sparged with argon in the shunt pathway

[k] The buffer was sparged with argon in the shunt pathway (H_2O_2) and the cofactor NADH was added

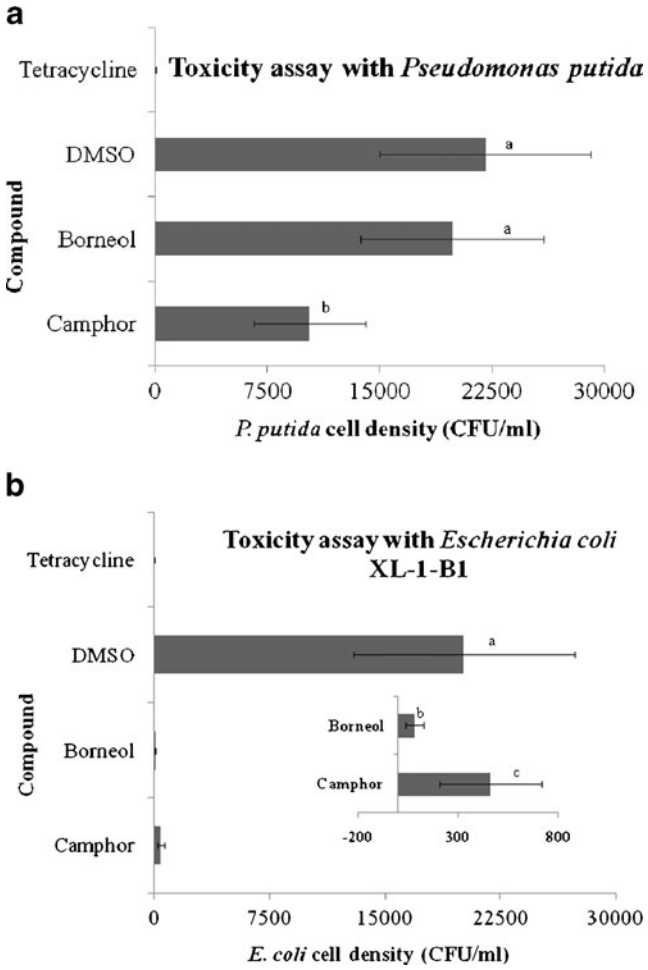


Fig. 4 Toxicity assays for camphor and borneol in two strains of bacteria: (**a**) ATCC of *Pseudomonas putida* and (**b**) XL-1-Blue of *Escherichia coli*. Histogram bars with different letters indicate significantly different mean cell densities (Tukey-Kramer pairwise comparisons, α=0.05)

forms during $P450_{cam}$ catalysis. The distribution of products appears to be dependent on the reaction conditions. Under well oxygenated conditions, 5-ketocamphor (**3**) is the major product, whereas under poorly aerated conditions borneol (**4**) is the major product. One way in which borneol could form is via reduction of the 2-keto group of camphor with NADH. Two observations conflict with this hypothesis. First, the formation of borneol also can occur in the absence of NADH, when shunting agents are used instead of O_2 as the oxidant. Second, NADH appears not to be necessary for borneol formation, as addition of NADD does not result in deuterated borneol (Rojubally, 2007).

Previous studies may have missed the formation of borneol, because various camphor-derived compounds that elute after camphor like borneol, isoborneol, and 5-*exo*-hydroxycamphor elute closely on non-polar GC columns typically used for this analysis. Furthermore, the mass spectra of the oxygenated bornyl-based monoterpenes (**1**, **2**, **3**, **4,** and **5**) are similar, because of a strong tendency to fragment to ions with *m/z* of 108, 95, 81, and 67. From studies with ^{17}O- labeled camphor, we know that none of these ions contain oxygen (Prasad et al., 2011). The products of camphor oxidation and the reduction product, borneol were detected in the early stages of metabolism, up to ~6 hr of culture. Further metabolism of borneol to 5-*exo*-hydroxy borneol (**12**) was observed in the non-aerated conditions, which suggests that the reduction to borneol and its further hydroxylation to (**12**) occurs in the intact bacterium. To test for a potential adaptive value for this reaction, we compared the relative toxicity of camphor and borneol on *P. putida* and on *E. coli.* This experiment suggested that camphor is toxic to both *E. coli* and *P. putida*, whereas borneol is more toxic than camphor to *E. coli*. Therefore, formation of borneol may confer an advantage to strains of *P. putida* that metabolize camphor and are competing with other bacteria that do not metabolize camphor. Under conditions of low oxygen, borneol forms from camphor, and this compound is less toxic than camphor itself to *P. putida* but more toxic to P450-free bacteria such as *E. coli*.

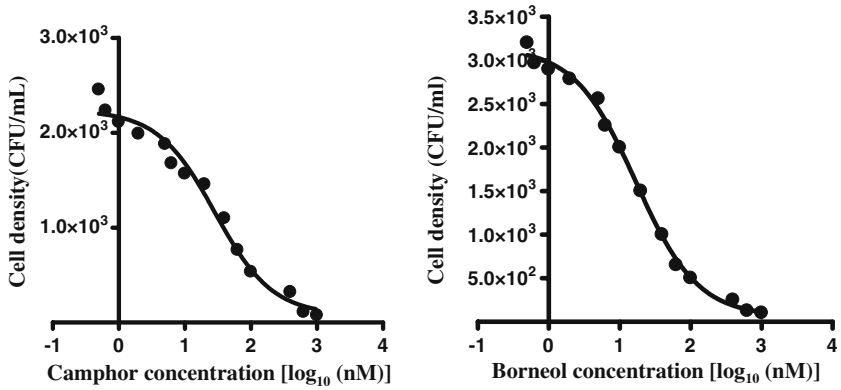


Fig. 5 IC50 experiments on *Escherichia coli* with varying concentrations of camphor and borneol

We are not aware whether *E. coli* and *P. putida* compete with each other ecologically.[1] We know that *P. putida*, for example, exists in soil and in sink sludge (Shen and Wang, 1995) (from where the camphor metabolizing strain of *P. putida* was originally isolated). *Escherichia coli* is a widespread enteric bacterium, but also can exist outside of vertebrate organisms (in soil or in water), as some past tragic incidents with drinking water in rural areas have demonstrated (Winfield and Groisman, 2003).

Bacterial P450's have been widely studied in the past for their ecological functions in the detoxification of both natural and artificial toxicants. For example, P450 BM3 from *Bacillus megaterium* hydroxylates fatty acids, fatty amides, and alcohols at the ω-1, ω-2, or ω-3 positions, making them less amphiphilic (Narhi and Fulco, 1986). P450 isolated from the actinomycete *Nonomuraea recticatena IFO 14525*, catalyzes the hydroxylation of oleanolic acid, a toxic triterpene (Kinoshita et al., 1999). P450s from members of the order *Actinomycetales* catalyze pentachlorophenol dehalogenation and the degradation of sulphonylurea herbicides (Munro and Lindsay, 1996).

In summary, we have identified the reduction product borneol by using the substrate camphor and purified *P. putida* proteins. We plan to perform experiments with the recombinant proteins expressed in *E. coli*. The source of electrons for this reduction reaction and our proposed mechanism for the reduction mechanism will be reported in the near future.

Acknowledgments This work was supported by NSERC (Discovery grant No. 222923-05). We thank the Chem 381 class (Spring 2006) for help in isolating products from *P. putida*. B. Prasad performed experiments with the purified $P450_{cam}$ system, with camphor/borneol toxicity, the IC_{50} experiments and wrote the paper. A. Rojubally performed the standard syntheses and time course experiments with *P. putida*. E. Plettner designed and planned the project.

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COMMENTARY

New Synthesis—Back to the Future: New Approaches and Directions in Chemical Studies of Coevolution

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The concept of an arms race between herbivores and plants, associated with increases in diversity of plant secondary compounds, has long provided an appealing theoretical framework for evolutionary biologists pondering adaptive radiations, and has been a stimulus for studies of chemical ecology. While publications on coevolution are steadily increasing in ecology and evolutionary biology, such studies have stagnated in chemical ecology, even though modern chemical techniques have made it easier to examine coevolutionary predictions for phytochemical diversification. An example of what can be accomplished is illustrated by Kursar et al. (2009), who documented considerable divergence in effective antiherbivore defenses of the tropical plant genus *Inga*, indicating that an arms race between plants and herbivores caused rapid, divergent trait evolution. Interestingly, closely related *Inga* species that occur together in tropical forests also have distinct defensive profiles driven by tritrophic interactions. Thus, diffuse multitrophic coevolutionary interactions, mediated in part by plant chemistry, help structure diverse communities, allowing for high alpha diversity for *Inga*.

The study of *Inga* and related studies on *Quercus*, *Piper*, and *Bursera* utilize modern approaches to natural history, ecology, phylogenetics, and chemistry, and indicate that the time is ripe for chemical ecologists to return to coevolution as a unifying research theme. For natural products chemistry, synthesis and complex mixture analysis have improved dramatically (Hoye and Sizova 2009) with advances in MS sources, including electrospray ionization, matrix-assisted laser desorption ionization, and atmospheric pressure chemical ionization. These approaches permit rapid elucidation of a broad array of compounds in a taxon of interest, which is necessary for testing causes and consequences of increases of chemical diversity. Such input from chemists can synergize with advances in molecular ecology. For example, new sequencing technologies present the opportunity to generate high resolution population genomic data that can be used to test the hypothesis that host plant affiliations are associated with distinct chemical profiles and population genetic differentiation.

How will these new technologies contribute to understanding chemical mediation of coevolution? The answer includes a long list of theoretical advances, but here I provide two examples of exciting research directions.

First, a major problem with any approach to studying insect-plant coevolution is that there is a bias towards examining individual plant compounds and their effects on the physiology of herbivores and their predators. Redundancy in plant defenses has long been appreciated, and common effects such as synergy obfuscate results from traditional mechanistic tests of coevolutionary hypotheses. For example, in numerous coevolutionary studies, the failure to demonstrate negative correlations in performance on hosts with different defensive compounds or to demonstrate defensive efficacies of secondary metabolites could easily be due to the fact that compounds do not function in isolation. It is quite possible that phytochemical synergy is the rule rather than the exception, even for putatively induced defenses, and we need new initiatives of chemical ecological research that aim to test for defensive efficacy of suites of compounds or cross-host tradeoffs for different synergistic mixtures.

Second, any new approach to studying coevolution must acknowledge that plants and parasites interact within complex food webs. Thus, it makes sense to examine the phylogenetic histories and ecological interactions of both the hosts and the parasites of focal herbivores. Forister and Feldman (2011) take this approach when they present the concept of a "phylogenetic cascade," in which diversification at one trophic level affects two or more other levels, resulting in a shared evolutionary history across a food chain. Classic coevolutionary hypotheses are a subset of the phylogenetic cascades framework, and chemistry can play a major role in these interactions, as evidenced from numerous ecological studies that demonstrate strong indirect trophic effects of variation in plant chemistry. If future research on chemical mediation of coevolution is considered within synergistic or phylogenetic cascades contexts, by utilizing the latest technological advances, our understanding of diversity of animals, plants, and secondary metabolites should improve dramatically.

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Differential Levels of Stress Proteins (HSPs) in Male and Female *Daphnia magna* in Response to Thermal Stress: A Consequence of Sex-Related Behavioral Differences?

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Abstract In two independent experiments, we compared: (1) water depth selection (and accompanying temperature selection) by male and female *Daphnia magna* under different kinds of environmental stress, including the presence of filamentous cyanobacteria, the risk of predation from fish, and the presence of toxic compounds; and (2) sex-dependent production of heat shock proteins (HSP60, 70, and 90) in response to a sudden change in temperature. Male *D. magna* selected deep water strata, which offer a relatively stable environment, and thereby avoided the threat of predation and the presence of toxic compounds in surface waters. Correlated with this behavior, males reduce their molecular defenses against stress, such as the production of heat shock proteins (HSPs), and do not maintain the physiological machinery that triggers an increase in HSP levels in response to stress. In contrast, female *D. magna* actively select habitats that offer optimal conditions for growth and production of offspring. Consequently, females are exposed to variable environmental conditions that may be associated with increased stress. To permit survival in these different habitats, *D. magna* females require molecular mechanisms to protect their cells from rapid changes in stress levels. Thus, they maintain high constitutive levels of the heat shock proteins from HSP 60, 70, and 90 families, and they have the potential to further enhance the production of the majority of these proteins under stress conditions. The results of this study indicate that the separate habitats selected by male and female *D. magna* result in different patterns of HSP production, leading us to hypothesize that that male and female *Daphnia magna* adopt different strategies to maximize the fitness of the species.

Key Words Biotic and abiotic stress · *Daphnia magna* · Heat Shock Proteins (HSPs) · SDS polyacrylamide gel electrophoresis (SDS-PAGE) · Sex · Temperature · Western blot

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Introduction

In many species, differences between males and females are not restricted to the morphology of sexual structures involved in mating or fertilization (Andersson, 1994). Individuals of each sex possess unique adaptations to maximize fitness. Under favorable environmental conditions, most water fleas, *Daphnia* spp. (Cladocera, Daphniidae) reproduce parthenogenetically. When conditions become adverse, *Daphnia* females produce resting eggs and deposit them into a special carapace structure called the ephippium. For successful hatching, resting eggs require fertilization before storage in the ephippium (Hobæk and Larsson, 1990; Pijanowska and Stolpe, 1996; Shurin and Dodson, 1997; Gyllström and Hansson, 2004).

The optimal strategy of female *Daphnia* involves a trade-off between the necessity to assimilate enough energy to grow and produce offspring, and the avoidance of environmental risks (e.g., predation or toxicity). The impact

of majority of stress factors is associated with surface water. Harmful chemicals that reach the lake in summer remain in the epilimnion (surface, warm water stratum). Even when these stresses reach the hypolimnion (deep, cold, and dark water stratum), their influence on organisms is limited due to low temperature (Cairns et al., 1975). Risk from predaceous fish that rely on vision to locate their prey, strongly depends on light and, as a consequence, is constrained to the upper strata. Because of the high temperatures and the abundance of food, epilimnetic conditions allow rapid growth (Wetzel, 2001). The necessity of avoiding the most dangerous locations at the surface and, at the same time, maximizing growth and reproduction has promoted the evolution of vertical migration behavior in *Daphnia* (e.g., Dawidowicz and Loose, 1992). Unlike females, males do not need to attain a large size or gather as much energy for reproduction. Their fitness depends on the number of females they fertilize during their lifetime. Therefore, their main goal is continued survival. We hypothesize that male *Daphnia magna* Straus should remain deeper in the water column than females, where they experience lower temperatures and food concentrations, but avoid predation by fish and adverse factors like cyanobacteria or toxins that are more prevalent in surface waters. Such male behavior has been described in *Daphnia galeata* by Spaak and Boersma (2001), who explained the inter-sexual differences in migratory behavior as a strategy to avoid inbreeding.

One of the main consequences of vertical migration in stratified lakes is exposure to rapid changes in temperature. For example, *D. magna* migrating twice a day – from the epilimnion to the hypolimnion and back – can experience a temperature gradient of 10-15°C. The reaction to thermal shock at the cellular level involves the expression of heat shock (or stress) proteins (HSPs) that protect cell structures against thermal stress (Jolly and Morimoto, 2000).

A change in the level of HSPs is one of the most widely recognized molecular responses to stress in pro- and eukaryotic organisms (Lindquist and Craig, 1988; Mager and de Kruijff, 1995; Jolly and Morimoto, 2000). HSPs were first discovered in *Drosophila busckii* following exposure to high temperature (Ritossa, 1962). They are divided into several groups (families) of different molecular weights (kDa): e.g., HSP100, HSP90, HSP70, HSP60, HSP40, and small proteins. HSP60s control modifications of newly synthesized proteins, repair damaged ones, and prevent peptides from accumulating (Jolly and Morimoto, 2000; Pockley, 2003). HSP70s are the most important molecular chaperones permanently active in the cell. In cooperation with other proteins, they are responsible for cellular homeostasis under non-stress conditions. Moreover, they play a vital role in stress tolerance and survival under adverse conditions. HSP70s also act to reduce accumulation of peptide aggregates, but if this does happen they can dissolve aggregates. HSP70s also promote the correct folding of newly synthesized proteins (Mayer and Bukau, 2005). HSP90s play a major role in stress tolerance. They act to remove proteins with incorrect structure, and by mediating proper folding under stress conditions, they prevent the accumulation of peptide aggregates. They also regulate the activity of other proteins (e.g., kinases) and stabilize the cytoskeleton (Csermely et al., 1998).

The induction of HSPs in *Daphnia* sp. was first described as a reaction to arsenic stress (Chen et al., 1999). Few studies have examined changes in HSP levels in response to biotic factors such as predation risk (Pijanowska and Kloc, 2004; Pauwels et al., 2005) and parasite infection (Pauwels et al., 2007). We recently have observed enhanced production of HSP60, HSP70, and HSP90 in heat-shocked *D. magna* (Mikulski et al., 2009). If the amplitude of migration of *D. magna* females is indeed broader than that of males, there are likely to be sex-related differences in the molecular response of HSPs to changes in temperature experienced by these animals.

The primary aim of this study was to examine whether depth (temperature) selection by *D. magna* in response to different kind of stress [the presence of cyanobacteria, fish, and polychlorinated biphenyls (PCBs) and their various combinations] differs between males and females. A second aim was to determine whether male and female *Daphnia* differ in the constitutive level of stress proteins and their production in response to changing temperature.

Methods and Materials

Two experiments were conducted on genetically identical individuals of a *D. magna* clone originating from Binnensee, a shallow coastal lake in northwestern Germany. To avoid any maternal effect, *D. magna* were cultured in 0.8 l of medium in glass jars (20 animals per jar) at 20°C under a summer photoperiod (16:8 hL:D) for several generations before the experiment. The experimental medium was lake water that had been aerated for a few weeks and filtered through a 0.2 μm capsule filter. Animals were fed green algae, *Scenedesmus obliquus*, at the non-limiting concentration of 1 mg C/l (Lampert, 1987), and the experimental medium was changed daily. Neonates from the second clutch were used for further breeding.

In the first experiment, differences in depth selection between *D. magna* males and females exposed to different kinds of stress were recorded. The stresses tested were the presence of filamentous cyanobacteria, the threat of fish predation, and the presence of toxic compounds, all of which are more severe in surface waters (reviewed by Mengel and Clarck, 1988). Moreover, all of these factors

are known to influence depth selection by cladocerans (Bernatowicz and Pijanowska, 2010). In the second experiment, the levels of HSPs were quantified in male and female *D. magna* exposed to thermal stress as a surrogate for depth selection.

Depth Selection of Males and Females A single cohort of *D. magna* neonates from the pre-experimental culture was transferred to several 0.8 l glass jars (20 animals of unknown sex per jar) and cultured at 20°C under a summer photoperiod (16:8 hL:D) for 6 d after birth. The animals were fed green algae, *Scenedesmus obliquus*, at 1 mg C/l, and the growth medium was changed daily.

On the seventh day, after all animals had reached maturity, some were selected for further experiments and transferred (3 females or 5 males per tube) to flow-through tubes (60 cm long, 1 cm diam) placed in a water bath, with surface waters heated to 22°C and bottom waters cooled to 9°C, and illuminated from above. This system, known as a "plankton organ," is widely used for studies on the diel vertical migration behavior of *Daphnia* (see Dawidowicz and Loose, 1992). The temperature gradient (Fig. 1) was stable throughout the experiment in all tubes. The specific stress factors then were applied, in four replicates (tubes) each, to both females and males: (1) the toxic cyanobacterium, *Cylindrospermopsis raciborskii* (Cy), from a laboratory culture added to the standard medium at a concentration of 1 mg C/l; (2) fish-conditioned (Fi) medium prepared by using water in which crucian carp, *Carassius carassius*, had been held for 24 h (1 fish per 10 l water); (3) toxic polychlorinated biphenyls PCB 52; and (4) PCB 153 added at a concentration of 10 μg/l. In the control treatment, standard medium containing only the *Scenedesmus obliquus* food was used. In addition to these single stressor treatments, 7 different combinations of these stress factors also were applied (see Fig. 2). The flow-through system ensured even distribution of food and chemicals in all tubes.

The depth of each animal in each tube was recorded at noon, for 2 d. From these data, the temperature experienced by individuals at particular depths was extrapolated (based on data shown in Fig. 1). The data were analyzed by using two-way nested ANOVA (with tube effect nested in treatment and sex as independent variables). Based on the mean temperature experienced by animals in the separate treatments, Levene's test of homogeneity of variance (Levene, 1960) was applied to compare the variability of temperature-depth selection between males and females exposed to the experimental factors.

Analysis of HSP Levels in Males and Females A single cohort of *D. magna* neonates from the pre-experimental culture was transferred to several 0.8 l glass jars (20 animals of unknown sex per jar) and cultured at 20°C under constant dim light until first reproduction. Animals were

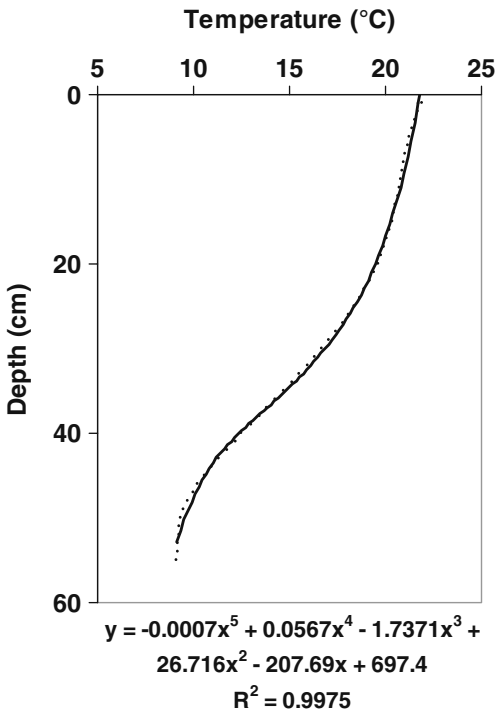


Fig. 1 The thermal profile in the experimental "plankton organ" tubes used to assay responses of *Daphnia magna* to various stress factors

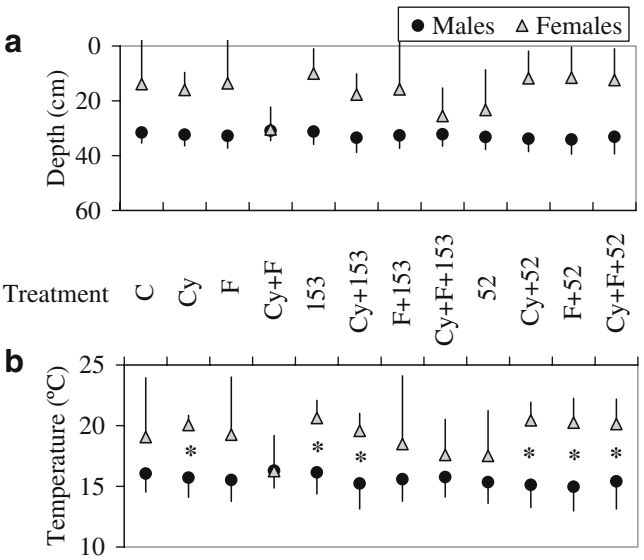


Fig. 2 The effect of different stress factors on **a** depth and **b** temperature selection (mean ± s.d.) by *Daphnia magna* males and females. C–control; Cy–cyanobacteria, *Cylindrospermopsis raciborskii*; F–fish, crucian carp, *Carassius carassius*; 153–toxic polychlorinated biphenyl (PCB)153; and 52–PCB52. * $P<0.05$ (significant difference between sexes in temperature selection within the same treatment; Tukey test, $N=4$)

fed green algae, *Scenedesmus obliquus*, at a concentration of 1 mg C/l and the growth medium was changed daily. After reaching maturity, animals were transferred to twelve glass jars (each treatment replicated three times) containing 200 ml medium (5 females or 15 males per jar). The combined mass of 5 females was approximately equal to that of 15 males, so the total mass of animals in each jar was roughly equivalent. After 24 h, half of the medium was removed from each jar. The same medium was immediately pumped back into 6 control jars (3 with males, 3 with females), while the other 6 jars were filled with hot medium to produce a 10°C increase in the water temperature within a few seconds. These jars were transferred to a 30°C water bath for 1 h. The range of temperature change was selected based on Mikulski et al. (2009). The thermal gradient set in this experiment (and the resulting thermal shock experienced by the animals) was more severe than in those conducted previously. Our unpublished data show that coping with even milder temperature gradients, e.g., crossing the thermocline (from 20°C at the surface to 15°C at the preferable depth), generates significant costs for female *D. magna* in terms of their life history and through enhanced production of HSPs. In this experiment, the severity of the thermal stress was increased to highlight the impairment of HSP production in males.

After this period of incubation at 30°C, the jars were removed from the water bath and slowly (over about 20 min) cooled to 20°C. Six h after the start of the heat treatment, the *D. magna* from each jar were collected, immediately dried on a sieve, and then transferred to 1.5 ml microcentrifuge tubes and homogenized in 75 μl of RIPA buffer (0.15 M NaCl, 1% deoxycholate Na salt, 1% Triton X-100, 0.1% SDS, 0.01 M Tris-HCl, pH 7.2) containing Complete protease inhibitors (Roche, Basel, Switzerland, Cat. No. 11836153001). The homogenates were centrifuged for 1 min at 13.4 rcf, and 10 μl aliquots of each supernatant were used to measure protein concentration with a DC Protein Assay (Bio-Rad, Berkeley, CA, USA, Cat. No. 500–0001) to ensure equal protein loading.

The remaining supernatants were transferred to fresh microcentrifuge tubes, and an equal volume of SDS-PAGE loading buffer (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. L4390) was added. After boiling for 5 min, samples containing equivalent amounts of protein (30 μg) were separated by electrophoresis on 25% SDS-polyacrylamide gels, and HSPs were immunodetected by western blotting with four different primary antibodies made by Stressgen (Victoria, British Columbia, Canada): Anti-Hsp60 (Stressgen, Cat. No. SPA-805 and Cat. No. SPA-807), Anti-Hsp70 (Stressgen, Cat. No. SPA-812) and Anti-Hsp90 (Stressgen, Cat. No. SPA-846). The blots then were incubated with alkaline phosphatase-conjugated secondary antibody (Anti-Rabbit IgG - Stressgen, Cat. No. 611–1502), and positive immunoreaction was identified by a color reaction with SIGMAFAST™ BCIP/NBT substrate (Sigma-Aldrich, Cat. No. B5655).

The processed blots were examined with a Bio-Rad Gel-Doc system, and the intensity of immunostained bands was determined by using Quantity One analysis software (Bio-Rad, Cat. No #170-9600).

The relative amounts of the individual HSPs in each sample were calculated as a percentage of the intensity of the corresponding HSP bands in a thermally stressed human HeLa cell positive control sample (Stressgen, Cat. No LYC-HL101) run alongside the test samples. Data were analyzed by using the ANOVA model and *post-hoc* Tukey test with Statistica (StatSoft, Tulsa, OK, USA).

The Anti-Hsp60 and Anti-Hsp70 antibodies revealed multiple immunoreactive protein bands of different molecular weights. These bands were treated as separate proteins, as described by Mikulski et al. (2009).

Results

Generally, the stress factors (toxic cyanobacteria, presence of fish, toxic polychlorinated biphenyls, or combinations of these cues) (ANOVA: $F_{(11,\ 288)}=2.24$, $P=0.013$) and sex (ANOVA: $F_{(1,\ 288)}=207.99$, $P<0.001$) influenced depth (temperature) selection of *D. magna* (Fig. 2). Males occupied colder (deeper) water strata than females (Tukey test: $P<0.001$). There was a strong interaction between sex and treatment effects (ANOVA: $F_{(11,\ 288)}=2.24$, $P<0.001$). The different types of environmental stress resulted in higher variability of depth (and temperature) selection in *D. magna* females than in males (Fig. 2; Table 1; Levene's test: $F_{(1,22)}=11.25$, $P=0.003$). Thus, males did not change their depth (temperature), but females varied their depth according to the nature of the environmental stress.

The pattern of production of different HSPs detected in *D. magna* (Mikulski et al., 2009) depended on the sex of the animal (Table 2, Fig. 3), and apart from the 79-kDa

Table 1 Temperature selection by male and female *Daphnia magna* exposed to different environmental stresses[a] and allowed to select their preferred depth in experimental tubes

Sex	Temperature (°C)		
	Min.	Max.	Variance
Male	14.96	16.29	1.01
Female	16.26	20.63	40.18

[a] Environmental stresses included exposure to toxic cyanobacteria, *Cylindrospermopsis raciborskii*; to water which held crucian carp, *Carassius carassius*; and to toxic polychlorinated biphenyls (see Methods and Materials)

Table 2 Sex, temperature, and interaction effects[a] on the levels of different HSPs in *Daphnia magna*

Molecular weight	Antibody	Factor	*df* effect	*df* error	*F*	*P*
50 kDa	Anti-Hsp60 (SPA-807)	Sex	**1**	**8**	**66.2**	**<0.001**
		Temperature	**1**	**8**	**14.3**	**0.005**
		Sex x Temp	**1**	**8**	**14.7**	**0.005**
55 kDa	Anti-Hsp60 (SPA-807)	Sex	**1**	**8**	**74.4**	**<0.001**
		Temperature	**1**	**8**	**13.3**	**0.006**
		Sex x Temp	**1**	**8**	**10.1**	**0.013**
73-74 kDa	Anti-Hsp70 (SPA-812)	Sex	**1**	**8**	**40.5**	**0.002**
		Temperature	**1**	**8**	**21.9**	**0.002**
		Sex x Temp	**1**	**8**	**19.0**	**0.002**
79 kDa	Anti-Hsp70 (SPA-812)	Sex	**1**	**8**	**42.3**	**<0.001**
		Temperature	1	8	1.7	0.224
		Sex x Temp	1	8	0.5	0.504
92 kDa	Anti-Hsp90 (SPA-846)	Sex	**1**	**8**	**164.1**	**<0.001**
		Temperature	**1**	**8**	**23.7**	**0.001**
		Sex x Temp	1	8	0.2	0.683

[a] Significant effects (ANOVA) are shown in bold-faced font

protein, the level of all HSPs was significantly higher in females than in males (Fig. 4). The level of the majority of HSPs (50 kDa, 55 kDa, 73–74 kDa, and 92 kDa) also was dependent on temperature (Table 2, Figs. 3 and 4). However, a significant increase in the level of these HSPs in response to raised temperature was only observed in females (see Fig. 3). There was a significant interaction between the effect of thermal shock and the effect of sex on the level of three HSPs: 50 kDa, 55 kDa, and 73–74 kDa (Table 2 and Fig. 3).

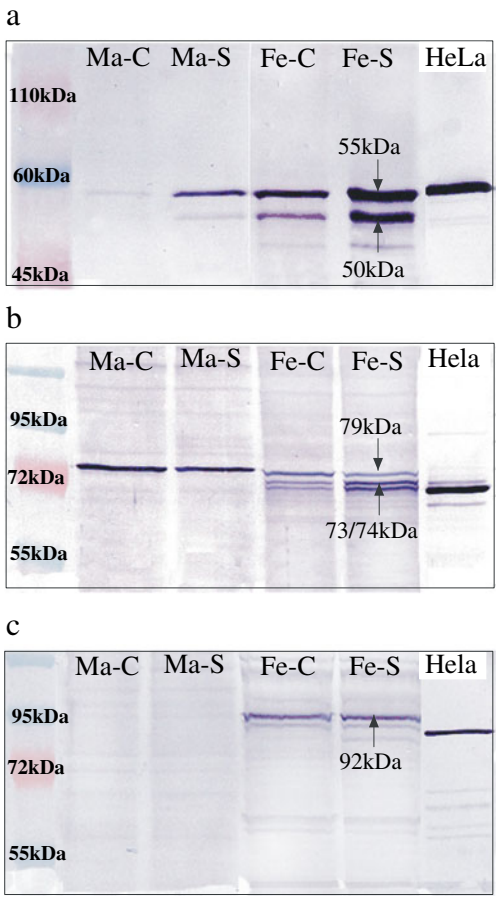


Fig. 3 Stress proteins in male (Ma) or female (Fe) *Daphnia magna* in control (−C) and heat-shock (−S) conditions revealed by anti-HSP60 (**a**), anti-HSP70 (**b**) and anti-HSP90 (**c**) antibodies, against heat-shocked human HeLa cell lysates

Discussion

According to our hypothesis, depth-temperature selection is more dependent on environmental stress in *D. magna* females than in males (Fig. 2). To achieve reproductive success, females must grow and assimilate sufficient energy. Therefore, they undertake vertical migration and adjust their depth according to the availability of food and the level of environmental stress (Dawidowicz and Loose, 1992). In contrast, the fitness of adult males seems to be poorly correlated with assimilation rate; rather, it would be expected to depend on their longevity, which is related more to safety than growth rate (Roff, 1992). Albeit smaller than females, size of males renders them susceptible to fish predation (e. g., Eggers, 1982). Therefore, males assimilate less energy and grow more slowly while staying in deeper water (poor in food but thermally stable), waiting for the appearance of receptive females. Since *D. magna* females are exposed to greater variability in environmental (including thermal) conditions than males, their molecular anti-stress machinery should be adequately developed.

We found that *D. magna* females maintain generally higher levels of HSPs than males (Fig. 4). Furthermore, a

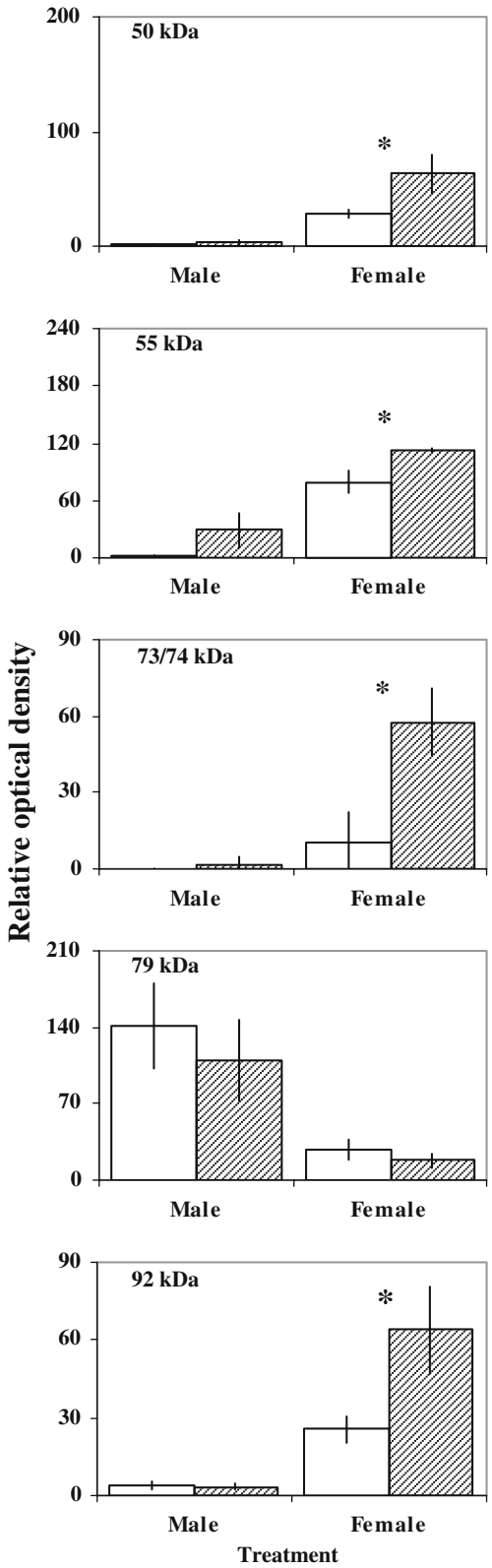


Fig. 4 The relative amounts (mean ± s.d.) of different HSPs expressed in control (white bars) and heat-shocked (shaded bars) male and female *Daphnia magna*. Optical density is expressed as a percentage of the density of the respective HSP bands in a thermally stressed human HeLa cell positive control sample run on the same gel; * $P<0.05$ (significant effect of heat shock on protein level, Tukey test, $N=3$)

significant increase in the level of these HSPs in response to thermal stress was observed solely in females (this inter-sex difference is supported by the significant interaction between the effect of thermal shock and the effect of sex on the level of the majority of heat shock proteins). Apparently, females that perform diel vertical migrations protect themselves against various stress factors associated with surface waters by maintaining a constitutively high level of HSPs and by greater responsiveness to temperature change. The only exception was HSP 79 kDa, which showed the opposite pattern, being more abundant in males than in females. It has been suggested that HSP 79 kDa represents a post-translational modification of HSP 73–74 kDa (Jolly and Morimoto, 2000). The intriguing physiological role of HSP70 in aquatic invertebrates is not well characterized. Higher levels of HSP70 in males compared with females previously have been described in *Drosophila,* although this apparently is not associated with greater survival in males (Dahlgaard et al., 1998). Only more detailed statistical analysis revealed a positive association between HSP70 level and survival after the induction of thermotolerance.

The results of our study show that behavioral and molecular defenses against environmental stress may be interrelated. *Daphnia magna* males can avoid dangers associated with surface waters by selecting the safe and stable (albeit cold with scarce food resources) environment of deep water strata. They may thus save energy by abandoning the expression of molecular defenses against stress. In contrast, *D. magna* females actively select an environment that is optimal for growth and reproduction, and, in consequence, are exposed to a broader range of environmental conditions that will unavoidably include stresses. For survival in these habitats, they require molecular mechanisms to protect their cells from adverse environmental conditions and rapid changes in stress levels. We have shown that these two strategies are adopted by genetically identical individuals of the same clone (sex in *D. magna* is environmentally determined; reviewed by Eads et al. 2008). The choice of both a behavioral strategy (migrating in the water column or permanently residing at certain depths) and a molecular strategy (e.g., activation of an inducible molecular anti-stress system) can thus constitute novel elements of phenotypic plasticity.

Although it is unwise to over-interpret the identification of these apparently linked defense mechanisms in a single clone (different genotypes are known to exhibit different stress responses), our study represents the first comprehen-

sive analysis of sex-related differences in the levels of stress proteins in *D. magna* in an ecological and evolutionary context.

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Up-Regulation of Lipoxygenase, Phospholipase, and Oxylipin-Production in the Induced Chemical Defense of the Red Alga *Gracilaria chilensis* against Epiphytes

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Abstract The red alga *Gracilaria chilensis* is commercially farmed for the production of agar hydrocolloids, but some susceptible algae in farms suffer from intense epiphyte growth. We investigated the induced chemical defense response of *G. chilensis* against epiphytes and demonstrated that an extract of an epiphyte-challenged alga can trigger a defense response. The hormonally active metabolites were purified by RP-HPLC. Treatment with the extract or the purified fraction changed the chemical profile of the alga and increased resistance against epiphyte spores. Semi-quantitative RT-PCR and enzyme assays demonstrated that this metabolic response occurs after an increase in lipoxygenase and phospholipase A2 activity. Although this suggests the involvement of regulatory oxylipins, neither jasmonic acid nor the algal metabolite prostaglandin E2 triggers comparable defense responses.

Key Words Induced defence · Red algae · Hormones · Epiphytism · Gracilaria chilensis · Bioassays

Introduction

For at least two decades, from 1980–2000, cultivation of the marine red alga *Gracilaria chilensis* for the production of agar hydrocolloids was a major asset of Chilean aquaculture. More than 300 farming operations were established across the country, with annual yields of over 120,000 wet metric tons (Buschmann et al., 2001). However, the decade 2000–2010 was marked by a dramatic decline in farming operations due to a combination of negative economical and biological factors. The main nuisance to farmers is the widespread occurrence of

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infections by algal epiphytes, which negatively affect the crop growth rate, lead to biomass losses due to increased drag, and result in products with lower economic value. In this context, we initiated a study to understand the biological and biochemical basis of the interactions between *Gracilaria chilensis* and its main epiphytic pests.

Resistance against epibionts could be mediated by either structural or chemical means, with both mechanisms interfering with the settling and penetration of spores (Wikstrom and Pavia, 2004; Nylund and Pavia, 2005; Paul et al., 2006). Direct morphological comparison and ultrastructural investigations of resistant and susceptible *G. chilensis* isolates revealed that resistance against epiphytes is not linked to any structural properties (Leonardi et al., 2006). Either constitutive or induced chemical defense is the most likely basis for resistance. Algae may store toxins or other adverse compounds and rely on the action of these constitutive defense metabolites (Paul et al., 2006; Nylund et al., 2010). If predation risk or pathogen pressure is highly variable, dynamic defenses may evolve that rely on the recognition of a challenge and a subsequent metabolic response (Pohnert, 2004; Weinberger, 2007).

Wound-activated defense is based on the rapid transformation of stored metabolites after tissue disruption, and results in reactive products that act with a very short delay (Pohnert, 2004). Recently, we found that *G. chilensis* reacts to tissue damage like that which occurs upon attack by certain epiphytes (Leonardi et al., 2006) with the release of hydroxylated and dihydroxylated fatty acids (Lion et al., 2006). If administered in artificial substrates in naturally occurring concentrations, these metabolites significantly inhibit the settlement of epiphyte spores. The biosynthetic pathway for these defense metabolites involves both the release of arachidonic acid from phospholipids and its subsequent oxidation to 8-hydroxyeicosapentaenoic acid (8-HETE), and the direct release of 7,8-dihydroxyeicosapentaenoic acid (7,8-diHETE) from galactolipids (Lion et al., 2006).

Induced defenses rely on signals from an attacker with the subsequent production of defense metabolites, a strategy that is well documented in higher plants (Baldwin, 1998; Howe and Jander, 2008). The mechanistic details of induced defenses in algae are different from higher plants (Gaquerel et al., 2007). Most of the research on induced defenses of algae has focused on herbivore-induced chemical defense (Pavia and Toth, 2000; Pohnert, 2004; Molis et al., 2006), but dynamic defensive processes also can be induced under the influence of endo- or epiphytes (Potin et al., 2002; Weinberger et al., 2002; Bouarab et al., 2004; Weinberger, 2007). We report here that, in addition to a wound-activated defense, an induced process is stimulated in *G. chilensis* by epiphyte attack. We found that immunity can be triggered by exposing *G. chilensis* to hormonally active metabolites prior to inoculation with epiphyte spores, and that the response needs several days for the full defensive potential to build up.

Methods and Materials

Algal Culture Unialgal *G. chilensis* C.J. Bird, McLachlan & E.C. Oliveira strains CR14 and CS7 (maintained in the culture collection at U. Catholica, Santiago, Chile), originating from an intensive mariculture farm at Caldera (Chile), were cultivated at 15°C and under a photon flux density of 40–50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (D12:L12) in 500 ml glass flasks containing SFC medium (Correa and McLachlan, 1991). The medium was changed weekly. Fertile fragments of *Acrochaetium sp.* and *Sahlingia subintegra* were isolated from infected thalli of *G. chilensis* collected at Caldera, Chile. Unialgal cultures of the epiphytes were maintained actively growing and reproducing by using standard culture conditions in enriched modified seawater medium SFC with 2 mM $NaNO_3$ and 100 μM NaH_2PO_4 but without Tris, Na_2SiO_3, or Mo, and with only 0.02 pM Co.

Preparation and Fractionation of Active Extracts A total of 1.2 kg wet weight of frozen *G. chilensis* that was previously exposed to epiphytes and subsequently cleaned mechanically to remove epiphytes was brought to room temperature, homogenized in artificial seawater with an ultra Turrax, and extracted three times with 1 L of CH_2Cl_2. Tissue disruption in seawater leads to elevated oxylipin levels (Lion et al., 2006). The organic phases were combined, filtered, and concentrated to about 200 ml. Excess water was removed by freezing and subsequently by drying over Na_2SO_4, and the remaining CH_2Cl_2 was removed in vacuum.

Fractionation of the crude CH_2Cl_2 extract was done by HPLC using a preparative Gilson 321 pump connected to a Gilson 115 UV-detector. Separation was achieved with a Merck LiChroCart 250-10 Purosphere RP-18 cartridge. A gradient of water (A) and acetonitrile (B) (0 min 100% A, 25 min 100% B, 30 min 100% B at a flow rate of 6 ml min^{-1}) was used for elution. Fractions corresponding to 0–40%, 41–60%, 61–80%, and 81–100% acetonitrile were collected, and the acetonitrile was removed by rotary evaporation. The remaining water was removed by freeze drying, and the resulting residue was dissolved in ethanol for further bioassays.

Monitoring Metabolic Responses Gracilaria chilensis plantlets that were about 10 cm in length were cultured at a plant density of 50 $g L^{-1}$ in Petri dishes on a shaker using culture conditions as described above. Plantlets were treated with solutions of the crude dichloromethane extract, the

respective HPLC fractions, 7,8-di-HETE, prostaglandin E2 (Sigma-Aldrich, Seelze, Germany), methyl jasmonate (Givaudan, Vernier, Swizerland), linolenic acid (Sigma-Aldrich), coronalone (Schuler et al., 2004), or pure EtOH (control). Stock solutions (10 mg ml^{-1} ethanol) were prepared with all samples except the HPLC fractions, for which the final concentrations were adjusted to give a concentration equivalent to 10 mg ml^{-1} of the crude extract used for separation. The final concentrations in the medium were adjusted to 100 μg ml^{-1} or the amount of the HPLC fraction that was obtained from 100 μg ml^{-1} crude extract. After incubation (18 h), the algal biomass was transferred to fresh medium and cultured for an additional 48 h. Samples for chemical analysis were frozen in liquid nitrogen and stored at −80°C until further work-up as described below.

Analytical Procedures For metabolic profiling, *G. chilensis* samples were frozen in liquid nitrogen, spiked with 1 mg ml^{-1} phenyldodecanoic acid in ethanol as an internal standard (400 μl g^{-1} alga), ground with mortar and pestle, and immediately extracted with methanol (10 ml g^{-1} alga). The extracts were concentrated under vacuum, redissolved in methanol (400 μL g^{-1} alga), centrifuged, and submitted to analytical HPLC-MS analysis on an Agilent series 1100 HPLC equipped with a RP18 normal phase column GROM-SIL 120 ODS3 CP, 125×2 mm, and a DAD detector connected to a Finnigan LCQ with an ESI interface (Thermo Finnigan, Manchester, UK). A gradient with 0.5% aqueous acetic acid (A) and acetonitrile (B) (0 min 50% A, 30 min 100% B, 35 min 100% B at a flow rate of 0.2 ml min^{-1}) was used for separation. Spectra were recorded in the positive mode, needle voltage 4 kV, capillary temperature 120°C.

Bioassays Precultivated apical fragments (1 cm long) of *G. chilensis* from two different isolates were placed in beakers with 60 ml SFC medium, and were treated with 10 μg ml^{-1} of dichloromethane extract, HPLC fractions in ethanol at 1 mg ml^{-1}, or solvent controls. Incubation times were 18 h or 18 h followed by a second incubation of 54 h in 60 ml fresh SFC medium without extract. After treatment, the fragments were rinsed, transferred to 60 ml of fresh SFC medium, and 20 fertile *Acrochaetium sp* thalli and a 2× 2 cm glass slide were added in order to quantify spore settlement on *G. chilensis* in comparison with the inert support. Settlement of epiphytic spores was recorded under a light microscope 48 h after the start of the co-culturing. Spores were counted on 1 cm transects of the alga and on identical transects of the control glass slide. For each replicate, 3 transects were counted. For each isolate, 5 independent replicates consisting of treatments and control were run. In all of the experiments, spore settlement did not differ between the two isolates ($P>0.5$), and thus the results were pooled and the 10 replicates statistically evaluated.

Native Polyacrylamide Gel Electrophoresis For protein extraction, ground samples of *G. chilensis* were incubated at 4°C for 60 min in 1 ml g^{-1} of extraction buffer (50 mM Tris–HCl, pH 9.5, 500 mM potassium chloride and 10 mM β-mercaptoethanol). Protein concentrations were determined according to the method of Bradford (Bradford, 1976). Crude protein extracts (80 μg of total protein per lane) were separated by polyacrylamide gel electrophoresis (PAGE) on 12% native polyacrylamide gels. Lipoxygenase (LOX) activity was stained with o-dianisidine (Delumen and Kazeniac, 1976) using Na-pyrophosphate buffer (0.1 M, pH 7.95) containing 1 mM KCN instead of Na-acetate buffer. The intensities of bands appearing in the presence of 2 mM arachidonic acid or linoleic acid were quantified by image analysis, using the SigmaScan Pro 5.0 software package (SPSS Inc., Chicago, IL, USA).

Putative LOX Gene Expression Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to analyze gene expression. RNA was isolated from *G. chilensis* that was ground in liquid nitrogen using the plant RNeasy kit (Qiagen, Hilden, Germany). To design homologous primers for RT-PCR of LOX transcripts, the amplification and cloning of a putative lipoxygenase (LOX) cDNA fragment from *G. chilensis* was undertaken using degenerate primers designed from available sequences of *Porphyra purpurea* [GenBank accession number U08842 (Liu and Reith, 1994)] and *Porphyra yezoensis* [Kazusa accession number AU186969 (Asamizu et al., 2003)] and from conserved sections of a putative LOX coding region selected in an unpublished EST database from *Gracilaria tenuistipitata* (Jonas Collén, personal communication). The two primers used were 5'-GATAAGGTTGGCGTGCAAGCA-3' and 5'-GTTTGGGACGTAGGACAAGTA-3'. The 740 bp RT-PCR product was cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced (GenBank accession number JF896804). RNA from *G. chilensis* treated with dichloromethane extract for 0, 6, and 24 h was pooled and amplified in a RT-PCR reaction. Three independent replicates were examined. Subunit I of cytochrome c oxidase (COX, GenBank accession number EF434915, forward: 5'-AAGTCACTCTGGTGGTGC-3' and reverse: 5'-GTATC-TACATCAAGACCTAC-3') was used as control. Reactions were carried out in a final volume of 25 μl of the following reaction mix: 1 μL cDNA, 1× GoTaq Reaction Buffer (2.5 mM $MgCl_2$) (Promega) with 0.2 mM dNTP, 1.25 units of GoTaq DNA polymerase (Promega) and 0.2 μM gene-specific primers (Eurogentec, Seraing, Belgium). PCR was performed on a GenAmp PCR System 2700 (Applied Biosystems Foster City, LA, USA). The cycling program for PCR was: initial denaturation 5 min at 95°C, 25 to 30 cycles of 15 s at 95°C, 30 s at 50°C, and 45 s at 72°C, and

final elongation for 3 min at 72°C. For each gene, 5 µl of PCR product were loaded on a 1.8% agarose electrophoresis gel and run at 100 V for 30 min before incubation with ethidium bromide (0.07 µg ml^{-1}). Images were obtained using Biovision 1000/26 M (Vilber Lourmat, Marne-la-Vallée, France), and one representative image of each replicate was selected for quantification. The expression levels were determined by quantifying the gel bands using ImageQuant TL software (Molecular Dynamics, GE Healthcare, Piscataway, NJ, USA). The relative changes in expression among genes were calculated as x-fold changes to the appropriate control treatments.

Quantification of PLA_2-activity Aliquots of protein extract containing 100 µg crude protein were diluted with protein extraction buffer to a volume of 500 µl. The fluorescent probe PED6 (Molecular Probes, Leiden, The Netherlands) was added from a stock solution in ethanol to a final concentration of 4 µg ml^{-1} PED6 and 0.4% ethanol. The mixture was incubated on a shaker for exactly 10 min at room temperature and in darkness. Enzymatic decomposition of PED6 was stopped by addition of 500 µl chloroform/acetic acid (200:1 *v/v*, pH 2.6). For extraction of the released fluorescent label (BODIPY® FL C_5), the mixture was incubated for 10 min on a shaker. After separation of phases, the organic phase was evaporated under reduced pressure at room temperature, and the residue was taken up in 250 µl ethanol. The fluorescence of 100 µl of the resulting extract was observed with a Wallac Victor^2 (Perkin Elmer, Courtaboeuf, France) plate fluorimeter (excitation wave length: 485 nm, emission wave length: 535 nm). The background fluorescence was examined in parallel experiments without the addition of PED6, and proved to be close to zero, due to the two step extraction in aqueous buffer and in chloroform, which eliminates all potentially interfering pigments.

Statistical Analysis Data were analyzed with the Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA). Data distributions were tested for normality and heteroscedasticity using the Shapiro-Wilk test ($P<0.05$) and Levene's test ($P<0.05$), respectively. The Student *t*-test ($P<0.05$) was used for comparisons among pairs of treatments, while 2-way-ANOVA ($P<0.05$) and the Tukey-*posthoc* test ($P<0.05$) were employed where interactive effects of treatment and incubation time had to be analyzed.

Results and Discussion

Field and laboratory studies suggested that resistance of *G. chilensis* towards epiphytic algae is dynamically regulated (Lion et al., 2006). We initiated a mechanistic study to look for mechanisms of dynamic defense responses in this alga. While an immediate production of defensive metabolites in *G. chilensis* is based on rapid enzymatic reactions that are triggered by wounding (Lion et al., 2006), induced defenses require more time and a complex cascade of signaling events that includes hormonal regulation of metabolic responses (Pohnert, 2004; Devoto and Turner, 2005; Conrath et al., 2006). To search for underlying principles of regulation in the induced defense of *G. chilensis* we assayed extracts derived from field samples of epiphyte-stressed algae for hormonal activity. Treatment of *G. chilensis* with a crude lipophilic extract from epiphyte-challenged conspecific algae induced a significant metabolic response (Fig. 1). In contrast, the aqueous extract from the same individuals was inactive (data not shown). Concentrations as low as 1 µg ml^{-1} of the CH_2Cl_2-extract of *G. chilensis* triggered an increase of 8-hydroxyeicosatetraenoic acid (8-HETE) and 7,8-dihydroxyeicosatetraenoic acid (7,8-di-HETE), two metabolites previously identified as involved in the activated defense of this alga against epiphytes (Lion et al., 2006). In addition, unidentified metabolites that were not found in unchallenged algae or wounded tissue were induced (Fig. 1). This demonstrates that the dichloromethane fraction triggers the production of secondary metabolites, and thus might contain a hormone involved in the up-regulation of pathways required to produce defense metabolites. Because we cannot culture epiphyte-free algae, we cannot conclude whether or not the production or the respective hormone(s) is induced by epiphytes. Future studies are required to clarify how this activity is regulated upon challenge of the alga.

Separation of the active extract by preparative HPLC yielded four fractions, and the less polar ones were tested. Only the fraction of medium polarity induced the production of secondary metabolites (Fig. 1). Neither the more polar nor the more nonpolar fraction triggered any metabolic response. We concluded there was activity associated with one specific metabolite or a group of metabolites with a narrow distribution of polarities (Fig. 1).

To gain further insight into the inducing metabolite(s) we evaluated the activity of candidate molecules identified in the active HPLC fraction of the *G. chilensis* extract, and also of known plant hormones. Treatments with 7,8-di-HETE and prostaglandin E2 did not change the metabolic profile of the algae. While 7,8-di-HETE is a specific metabolite found only in *G. chilensis*, prostaglandin E2 is a mammalian hormone that also occurs in several *Gracilaria spp.* including *G. chilensis* (Fusetani and Hashimoto, 1984). In addition, we tested methyl jasmonate, a plant hormone that was not detected in *G. chilensis* but was active in the red alga *Chondrus crispus* (Farmer and Ryan, 1990; Bouarab et al., 2004); coronalone, a synthetic analogue of jasmonic acid that is also hormonally active in plants (Schüler et al. 2004); and linolenic acid, the biosynthetic precursor of jasmonic acid. None of these potential hormones or hormone

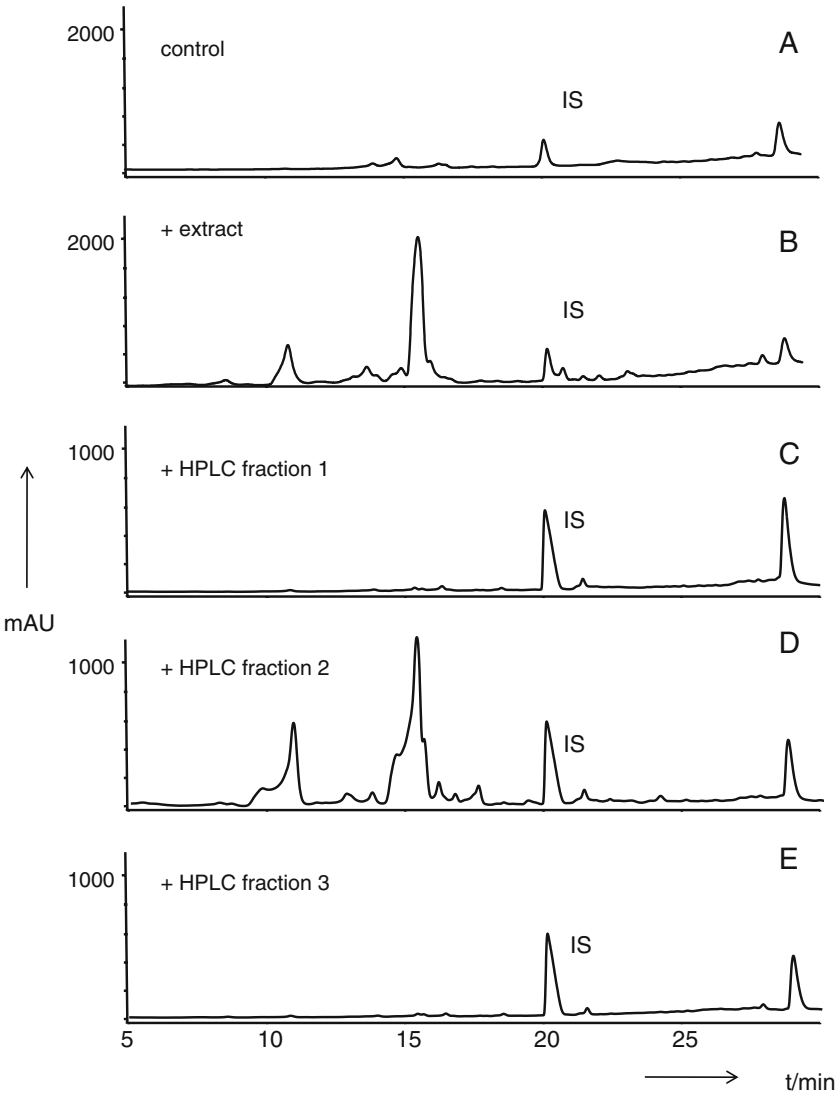


Fig. 1 Elicitation of the formation of oxylipins in *Gracilaria chilensis*. **a** Ethanol (control). **b** Crude dichloromethane extract (1 μg ml^{-1} in ethanol). The major peak at the retention time 11 min corresponds to 7,8-di-HETE and at 15.8 min to 8-HETE. **c**–**e** Fractions obtained by preparative RP-HPLC of the dichloromethane extract. Fractions eluted at 40–60% CH_3CN (**c**); 61–80% CH_3CN (**d**); 81–100% CH_3CN (**e**). The UV-trace at 254 nm is shown. Chromatograms were referenced in comparison to the internal standard (IS) phenyldocecenoic acid (retention time 20.5 min). Note the different scales of A, B and C-E

precursors triggered any detectable metabolic response in *G. chilensis* (data not shown). Concentrations of the plant hormones and coronalone that were tested exceeded those required to induce metabolic responses in higher plants and algae by about 5 to 50 fold, thus leading us to conclude that lack of activity was not a consequence of low concentration of the potential inducers (Bouarab et al., 2004; Schüler et al. 2004). The chemical nature of the active algal compound(s), which co-elutes with dihydroxylated C20 fatty acids in RP-HPLC, thus, remains unknown.

To determine whether the metabolic reaction observed after treatment with the crude, hormonally-active fraction is linked to an induced defense, we developed a bioassay in which settlement of epiphyte spores on induced and uninduced *G. chilensis* was monitored. In this assay, *G. chilensis* was incubated with fertile spores that were releasing *Acrochaetium sp.* thalli, and after defined time spans, the number of settled epiphyte spores on the algae were compared to the number of spores on a comparable

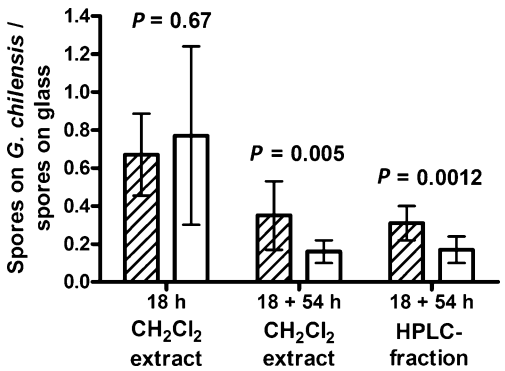


Fig. 2 Resistance of *Gracilaria chilensis* against spore settlement of the epiphyte *Acrochaetium* after different times of treatment with crude (10 μg ml^{-1}) or fractionated (corresponding to 10 μg ml^{-1} crude extract) CH_2Cl_2 extract in ethanol (*white bars*) or with ethanol only (*hatched bars*). Mean +/− standard deviation, N=10 (P values are indicated above bars)

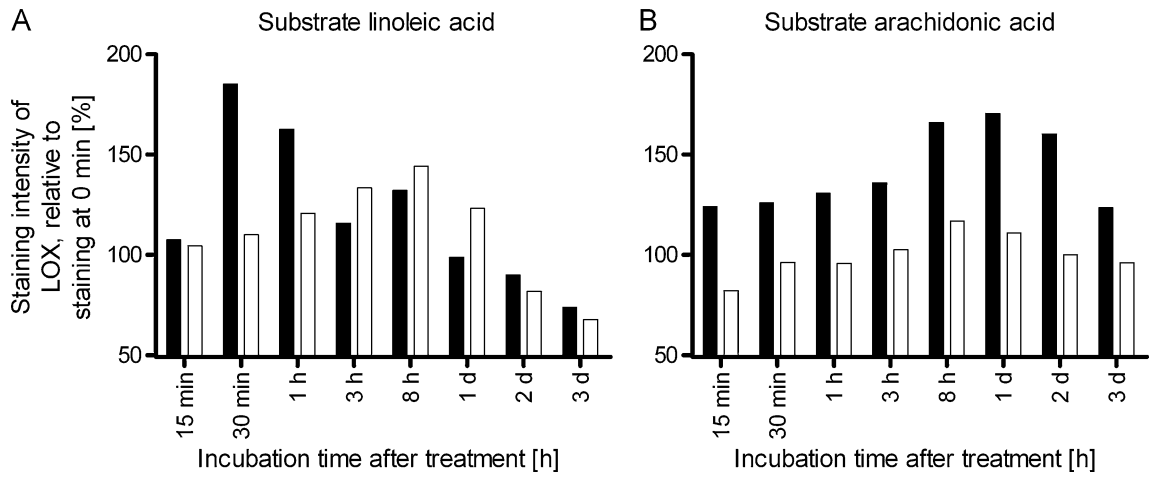


Fig. 3 Induction of lipoxygenase-type activity in *Gracilaria chilensis*. The activity was determined in native PAGE gels using 2 mM arachidonic acid or linoleic acid as a substrate. The protein extracts from algae that had been exposed to ethanol containing (*black bars*) or not containing (*white bars*) crude CH_2Cl_2-extract of *G. chilensis* for the times indicated

transect of a glass slide. This method allows normalization of the assays without the need to co-incubate treated and untreated *G. chilensis*, which could result in cross talk between the algae. Pre-treatment with the active extract for 18 h followed by a 54 h period in which the algae were allowed to develop metabolic reactions in pure culture medium resulted in a significantly reduced number of settled spores on the *G. chilensis* surface compared to algae that were treated with the solvent alone (P=0.005)(Fig. 2). The medium polarity HPLC fraction that induced a metabolic response also triggered a significant (P=0.001) reduction in the number of settled spores (Fig. 2). Resistance was observed only if an initial 18 h treatment with the active fraction was followed by a 54 h incubation period. Shorter incubations did not increase resistance. Results obtained with two different isolates of *G. chilensis* were statistically indistinguishable. Comparison of the uninduced isolates as controls also revealed no difference (P=0.63) in spore settlement. This significant response demonstrates clearly that a defense reaction against epiphyte spores can be triggered by algal compounds. Since shorter times of pre-incubation did not result in any significant biological response, it is likely that a slow up-regulation in defense genes is required for the establishment of the resistance. Comparable slow responses are known from the induced chemical defense of both plants and algae (Toth and Pavia, 2000; De Vos et al., 2005). It is important to note that the observed induced response is not a general defense against epiphytes, since parallel infection experiments with the red alga *Sahlingia subintegra* did not reveal any increased resistance (data not shown).

Recent work in algal chemical ecology suggests that induced defenses are widely distributed among seaweeds (Coleman et al., 2007; Weinberger, 2007; Molis et al., 2008; Rohde and Wahl, 2008). Nevertheless, besides studies on the activity of oxylipins from *Chondrus crispus,* little information about the nature of the involved signals is available (Bouarab et al., 2004; Gaquerel et al., 2007). Several hormones from a wide range of phyla from mammals to plants belong to the class of oxylipins. Since our HPLC fractionation of the crude extract also pointed

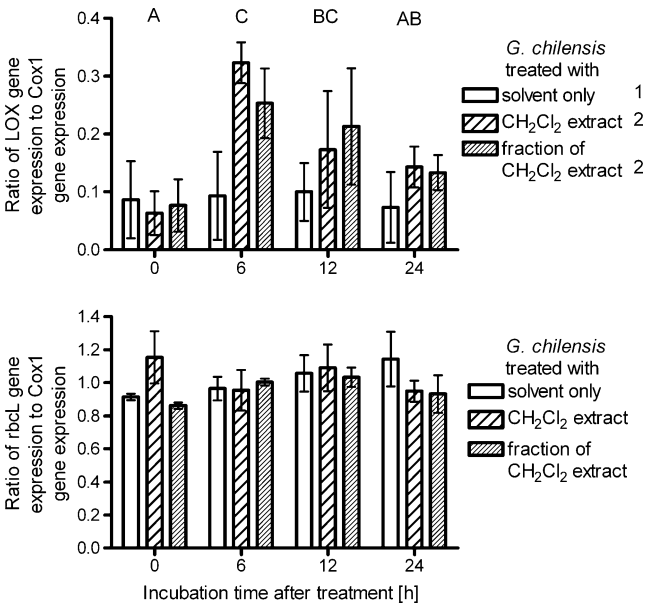


Fig. 4 Expression of a putative lipoxygenase gene in *Gracilaria chilensis* after treatment with dichloromethane extract. RNA from *G. chilensis* treated with solvent (control), with the dichloromethane extract or with fractions . Expression of LOX, the large subunit of ribulose-bisphosphate carboxylase (rbcL), or cytochrome c oxidase (COX) was quantified by semi-quantitative RT-PCRMean +/− standard deviation, N=3. Different capitals and different numbers indicate incubation times and treatments, respectively, that are significantly different (Tukey-test, P<0.05)

Table 1 Factorial ANOVA of (A) LOX to COX1 gene expression ratios and (B) rbcL to COX1 gene expression ratios in *Gracilaria chilensis* at four different times after treatment with CH_2Cl_2 extract, with a HPLC fraction of this extract or with solvent only. Incubation time and type of treatment were used as factors

Source	Sum of squares	Degrees of Freedom	Mean squares	*F*	*P*
A.					
Intercept	0.751111	1	0.751111	190.6911	0.000000
Time	0.108511	3	0.036170	9.1829	0.000316
Treatment	0.056939	2	0.028469	7.2278	0.003492
Time*Treatment	0.055706	6	0.009284	2.3571	0.062289
Error	0.094533	24	0.003939		
B.					
Intercept	33.78692	1	33.78692	3960.108	0.000000
Time	0.04101	3	0.01367	1.602	0.217416
Treatment	0.03413	2	0.01706	2.000	0.159185
Time*Treatment	0.15163	6	0.02527	2.962	0.028328
Error	0.18770	22	0.00853		

towards an active fraction that co-elutes with major C20-oxylipins from *G. chilensis*, such as 7,8-di-HETE, we decided to investigate the activity of key enzymes involved in the formation of these metabolites.

In-gel activity staining revealed that crude protein extracts of *G. chilensis* had lipoxygenase-activity towards linoleic and arachidonic acid (Fig. 3). Treatment of *G. chilensis* with dichloromethane-extract increased the algal capacity to oxidize linoleic acid within 30 min to 1 h after exposure (Fig. 3). The ability to oxidize arachidonic acid increased within 15 min of exposure, and was most substantially visible 8 h to 2 d after exposure (Fig. 3). The activity was insensitive to KCN, and was, therefore, not attributed to peroxidases or other heme proteins. but to lipoxygenases (Manchenko, 1994). Over the entire assay period, the up-regulation of linolenic acid LOX activity was not significant ($P>0.5$). When linoleic acid was used as substrate, only a weakly stained band was detected, although a relatively large amount of crude protein was loaded (80 μg per lane). The expression of a linolenic acid lipoxygenase was stronger in *C. crispus*, where more intense staining and two additional isoforms were detected on electrophoretic gels when only 30 μg protein were loaded (Bouarab et al., 2004). When arachidonic acid was used as the substrate during the gel staining procedure, a single stained band was detected accompanied by changes in arachidonic acid LOX activity upon challenge with the active extract ($P<0.001$). The stained band had a retention factor similar to the band observed after treatment with linoleic acid. However, the transformations were not catalyzed by the same enzyme because there were clear differences in the time courses of fatty acid hydroperoxide formation (Fig. 3). While induction with the dichloromethane extract resulted in a maximally increased arachidonic acid LOX activity after 24 h, linoleic acid was preferentially acted on after 30 min. The delayed metabolic response observed after the treatment with the extract is in accord with the delayed defense response in our bioassays. Such orchestrated expression of different lipoxygenases often is observed in higher plants, where oxylipins also play

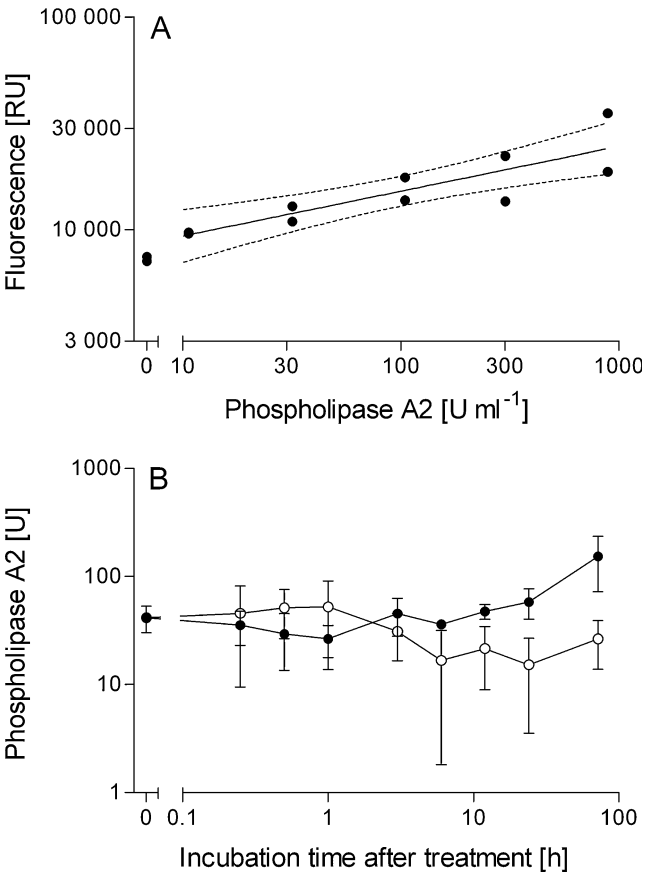


Fig. 5 Induction of phospholipase A2 in *Gracilaria chilensis*. **a** Calibration: Incubation of PED6 with increasing amounts of phospholipase A2 in 500 μl protein extraction buffer ($r^2=0.7366$, $P=0.002$ the dotted lines represent 95% confidence intervals). One U of enzyme hydrolyzes 1 μM phosphatidylcholine per min. **b** PLA2 activity in 100 μg crude protein originating from *G. chilensis* exposed (●) or unexposed (○) to dichlormethane extract. Averages of two replicates, bars represent minimum and maximum response

key roles in quick wound-activated and in slower induced defense reactions (Halitschke et al., 2000; Truman et al., 2007; Bonaventure and Baldwin, 2010).

The design of a set of primers based on an EST clone from a cDNA library of the red alga *Gracilaria tenuistipitata* (P. Nyvall, J. Collén et al., unpublished) allowed the cloning of a partial cDNA fragment from *G. chilensis*. This 764 bp fragment exhibited significant similarity to plant and animal LOX as well as to a putative red algal LOX (Supplemental Information). The corresponding amino acid sequence was 43% identical to the putative LOX from *Porphyra purpurea* (GenBank™ accession no. AAA61791). Sequence analysis shows that some of the residues that are involved in iron-ligand binding in all LOX (Prigge et al., 1997) are present in the partial sequence. Conserved sequences include the central histidine-rich region around His-510 and His-515 in the *P. purpurea* sequence, whereas the other residues including a distal histidine (His-638) and an asparagine (Asn-642) are missing in the partial fragment. The partial cDNA clone shared 87.4%, 52.2% and 50.6% similarity with the LOX cDNAs of *G. tenuistipitata* (J. Collén, pers. comm.), *Porphyra purpurea* (Liu and Reith, 1994), and *P. yezoensis* (Asamizu et al., 2003). The translated sequence also contained two determinants for substrate and regiospecificity (Sloane et al., 1991; Borngraber et al., 1996) as well as a generally conserved domain in lipoxygenases (Marchler-Bauer et al., 2005).

Expression of lipoxygenases was monitored by using semi-quantitative RT-PCR. A pronounced transcription of lipogygenase relative to cytochrome oxidase 1 was observed in *G. chilensis* after treatment with either the CH_2Cl_2 extract or the medium polar HPLC fraction (two-way-ANOVA and Tukey-test, $P<0.05$, Fig. 4, Table 1A). There were no changes in the expression of ribulose bisphosphate carboxylase relative to cytochrome oxidase 1 in the same samples, thus indicating a specific LOX upregulation (two-way-ANOVA and Tukey-test, $P>0.05$, Fig. 4). The effect was maximal after 6 h and still significant after 12 h, a delay that corresponds to the observed kinetics of the arachidonic acid LOX activity (Fig. 3). Only two-way ANOVA (Table 1B), but not the Tukey-test ($P<0.05$) detected a significant interaction of treatment and incubation time. The detected mRNA, therefore, probably encodes the enzyme that acts on arachidonic acid. To what extent the enzyme has a role in the direct synthesis of defensive oxylipins (Lion et al., 2006) vs. the production of regulatory active oxylipins can only be concluded indirectly. Since a further delay in the induced defense response is observed it might be more likely that LOX is involved in the triggering of further defensive metabolic reactions, also a well known phenomenon in higher plants.

Other enzymes that are crucial for the production of oxylipins are lipases. These enzymes release free unsaturated fatty acids, which are the substrates for LOX. They often are involved in the regulation of defense reactions in algae (Lion et al., 2006) and higher plants (Roy et al., 1995; Kallenbach et al., 2010). Treatment of *G. chilensis* with the dichloromethane extract also increased PLA_2 activity. In protein extraction buffer, enzyme activity could be quantified by the concentration-dependent release of fluorescent BODIPY FL C_5 from the phospholipase substrate PED6 (Fig. 5). Algae treated with dichlormethane extract for 6 h to 3 d had more activity than untreated plants (2-way-ANOVA, $P=0.03$). The strongest upregulation was observed after 3 d, when treated plants exhibited 5.8 times more PLA_2-activity than untreated plants. From the data on enzyme activity, we conclude that induction results after 6 h in an increased liberation of fatty acids from phospholipids, which probably fuel the lipoxygenase that is increasingly expressed after 8 h. To fully develop the defense potential the alga, however, requires additional time, since a response in the bioassay is delayed for another 48 h.

In summary, we provide evidence for an induced chemical defense mechanism of *G. chilensis* against epiphytes. This can be triggered by a hormonally active fraction of the *G. chilensis* extract, thereby supporting a general role of hormones in the induced defense of the alga. In addition, candidate genes and enzyme activities, commonly associated with an oxylipin based hormonal regulation of metabolic processes, are triggered by the addition of the active fraction. Further work will focus on the structural elucidation of the actual hormone and on an examination of how this hormone is regulated in response to biotic stress.

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Searching for Major Urinary Proteins (MUPs) as Chemosignals in Urine of Subterranean Rodents

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Abstract Chemosensory information mediates behavior in many rodent genera. Major Urinary Proteins (MUPs) facilitate chemical communication in some species of mice. We sought to demonstrate the importance of MUPs in chemosignaling across a range of rodent genera that live in different habitats and social structures. We analyzed urine from three subterranean rodent genera from different continents, and with diverse social systems: eusocial Zambian mole-rats (*Fukomys*), solitary Israeli blind mole rats (*Spalax*), and social Chilean coruros (*Spalacopus*). 2D gel electrophoresis revealed low levels of protein, with sequences similar to aphrodisin, in *Fukomys* mole-rat urine, but no MUPs in urine of any of the studied species. Previous research demonstrated that subjects from the tested genera responded differentially to odors indicating transmission of individuality, family/colony or population, species, and reproductive state in secretions and excretions of conspecifics. This extends, to subterranean rodents, the evidence that rodent species can successfully transmit and receive chemosignals without the necessity of MUPs.

Key Words Aphrodisin · Lipocalin · Major urinary proteins · Subterranean rodents · Mole-rat · Coruro · *Fukomys* · *Spalacopus* · *Spalax*

Introduction

Rodents depend on chemical secretions and excretions to communicate everything of social importance: their individual identity, sex, age, health, reproductive and social status, etc. (Brown and MacDonald, 1985). There is abundant behavioral evidence (reviewed, e.g., in Todrank and Heth, 2003) of adaptive responses in a broad range of rodent species to individual genotypes and changing biological states expressed in rodent semiochemicals. The processes underlying transmission of these complex, largely volatile, semiochemicals remain enigmatic. In particular, in rodent urine it is unclear to what extent urinary proteins are involved. Major Urinary Proteins (MUPs), with or without their bound ligands, are conduits of chemosignals in house mice (e.g., Beynon and Hurst, 2003, 2004; Cheetham et al., 2007), but studies of MUPs in wild rodents are scarce.

MUPs are expressed in the liver, filtered by the kidneys, and excreted in the urine, at quantities constituting up to 99% of protein content in adult male house mice (Humphries et al., 1999). The β-strands of lipocalins form a hydrophobic pocket in which small, volatile and non-polar molecules can be bound (Lücke et al., 1999; Cavaggioni and Mucignat-Caretta, 2000). MUPs have

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mass ranges of approximately 19 kDa (Robertson et al., 2007). Aphrodisin, another member of the lipocalin superfamily, with a mass of approximately 17 kDa (Henzel et al., 1988), originally was identified in the vaginal discharge of hamsters (Singer et al., 1986, 1987), but also has been found at low concentrations in the urine of hamsters (*Phodopus roborovskii*) and bank voles (*Myodes glareolus*) of both sexes (Beynon et al., 2008), along with three other aphrodisin-like odor-binding proteins (Stopková et al., 2010).

Because initial studies showed high polymorphism in MUPs (Robertson et al., 1996), it was concluded that variants in proportions of particular MUPs contribute inherently to signaling individuality in urine scent marks (Hurst et al., 2001). Findings from a few studies seem to support direct involvement of MUPs in conveying identity, species, and biological state (e.g., Mucignat-Caretta et al., 1995; Beynon et al., 2002; Nevison et al., 2003; Martìnez-Ricòs et al., 2007), while findings from other studies have suggested an indirect role of MUPs through active ligand binding or ligand/MUP-complexes (e.g., Novotny et al., 1999; Novotny, 2003; Peele et al., 2003).

Investigating the prevalence and modulation of MUPs across Rodentia could broaden the understanding of the function of MUPs in chemical communication. We hypothesized that if MUPs serve a central function in rodent chemical communication, they will be present, in abundance, across a broad spectrum of species, irrespective of habitat or social structure. Thus, in this study we looked for MUPs in urine samples from species that respond differentially to urine and bodily secretions of conspecifics (Todrank and Heth, 1996; Heth and Todrank, 2000; Heth et al., 2004; Hagemeyer and Begall, 2006; Hagemeyer, 2010), and which belong to three different subterranean genera, representing different rodent families and diverse social systems: eusocial Zambian mole-rats (*Fukomys*), solitary Israeli blind mole rats (*Spalax*), and social Chilean coruros (*Spalacopus*).

Methods and Materials

Animals We analyzed urine from 63 animals, representing three genera of subterranean rodents, for comparison with urine from C57BL/6 laboratory mice (Table 1). Eusocial Zambian *Fukomys* mole-rats (*Fukomys anselli*, *F. kafuensis*, and their hybrids, *F. anselli* x *F. kafuensis*) live in large families, consisting of a single reproductive pair and their philopatric offspring (cf., Begall and Burda, 1998; Burda et al., 2000). Israeli *Spalax ehrenbergi* blind mole rats (*S. galili, S. golani, S. carmeli*, and *S. judaei*) live solitarily in tunnel territories (Nevo et al., 2001). Social Chilean *Spalacopus cyanus* coruros live in harems (Begall et al., 1999).

Coruros (N=6 males, N=2 females) and *Fukomys* mole-rats (N=7 males, N=9 females), either wild-captured or laboratory-bred, were kept in an animal room at the Department of General Zoology in Essen (Germany), under a natural daylight cycle at room temperature (20–25°C) in glass terrariums of varying size (depending on colony size), with a floor consisting of a 5 cm layer of horticultural peat. They were provided with a diet of fresh carrots, potatoes, and apples, supplemented weekly with lettuce and cereals. The *Spalax* blind mole rats (N=16 males, N=9 females) were captured in the field and kept in the Institute of Evolution at the University of Haifa. Each of the 4 species was kept in a separate room under a 14:10 hr light:dark cycle with constant temperature (21±1°C) and relative humidity (70±5%). The animals were kept individually in plastic cages (30×40×25 cm) and were provided carrots

Table 1 Sample sizes, sex, age class, origin, and number of specimens with MUP/lipocalin-bands in SDS-PAGE

Species	Sample size	Sex		Continent (country)	Specimens with bands
		m	f		
Fukomys anselli (Bathyergidae)	2 kings, 3 queens, 7 non-reproductives	4	8	Africa (Zambia)	12
F. kafuensis (Bathyergidae)	1 king, 1 queen, 4 non-reproductives	2	4	Africa (Zambia)	6
F. anselli x *F. kaufensis* (Bathyergidae)	3 kings, 3 queens, 5 non-reproductives	7	4	Africa (Zambia)	11
Spalacopus cyanus (Octodontidae)	8 adults	6	2	South America (Chile)	0
Spalax galili (Spalacidae)	5 adults	3	2	Eurasia (Israel)	0
S. golani (Spalacidae)	2 adults	1	1	Eurasia (Israel)	0
S. carmeli (Spalacidae)	8 adults	6	2	Eurasia (Israel)	2 (m)
S. judaei (Spalacidae)	10 adults	6	4	Eurasia (Israel)	0
Mouse (Muridae)	1 adult		1	Eurasia (Czech Rep.)	1
Total	63 adults	32	21		

m males; *f* females

and potatoes *ad libitum*. Subterranean rodents do not drink free water, but get it from their food.

Laboratory mice (N=9 males, N=10 females), used as a reference species for the analysis, were kept under standard laboratory conditions at the Chair of Zoology of the Charles University in Prague, and provided water and commercial mouse pellets *ad libitum*.

Urine Collection Urine of *Fukomys* mole-rats was collected by picking up an animal by its back, gently, and putting a 1.5 ml Eppendorf-tube under the urogenital region. In rare instances when an animal did not urinate within 1 min, it was put into a clean bucket and allowed to urinate. Immediately after urination, the urine was transferred by pipette into an Eppendorf tube. This latter method was used with the coruros, as they seldom urinated when picked up. Urine samples were centrifuged at 8,000 rpm for 8 sec, and the supernatant transferred into a sterile Eppendorf tube. Following this procedure, urine samples were frozen at −80°C until analysis. Urine from *Spalax* mole rats was collected by picking up an animal and massaging the hind quarters until it urinated into a sterile plastic cup (from which the urine was transferred to sterile Eppendorf tubes and frozen at −80°C for shipment to Prague). Urine from mice was obtained by gently massaging the abdomen of an animal until it urinated into a 1.5 ml Eppendorf tube, which was placed immediately at −20°C.

Bradford Test and Creatinine Determination Total protein concentration in urine was measured by the Bradford method, using bovine serum albumin at known concentrations to create a standard curve (R^2>0.98) (Quick Start™ Bradford Protein Assay; BioRad Laboratories, Hercules, CA, USA).

To take different urine concentrations into account, the total protein concentration of each sample was corrected for creatinine (Cr) concentration. Creatinine is a breakdown product of tissue proteins, usually formed in muscles. For each sample, the Cr concentration was determined using the Jaffe reaction. The freshly prepared picric reagent (4 units sodium hydroxide, 1 unit picric acid) was incubated for 15 min. at room temperature in darkness (Bio Vender Lab., Brno, Czech Republic). In a 1 ml cuvette, 50 µl of standard, water (blank), or sample, were mixed and measured spectrophotometrically, at 492 nm, after 30 and 90 sec. We used the following formula to calculate the molar Cr concentration:

$$C_{creatinine} = \frac{C_{st} \cdot \left(\Delta A_{sample} - \Delta A_{blank}\right)}{\Delta A_{standard} - \Delta A_{blank}} [\mu\text{mol/l}]$$

C_{st} = concentration of standard (337 µmol/l, 0.038 mg/ml), A = absorbance, and delta A = (A after 90 sec-A after 30 sec). Results are presented as mg protein/mg Cr.

SDS-PAGE (Sodium Dodecylsulfate-Polyacrylamide-Gel-Electrophoresis) Urinary proteins were precipitated by pipetting 100 µl urine into 500 µl acetone, and then centrifugation at 14,000 g for 5 min. After this, samples were dissolved in 15 µl sample buffer [3.55 ml H_2O, 1.25 ml 0.5 M Tris/HCl (pH 6.8), 2.5 ml glycerol, 2.0 ml 10% SDS, 0.2 ml 0.5% bromphenol blue]. We used a modified protocol, based on that of Laemmli (1970). Samples were applied to 4–15% and 4–20% SDS-PAGE discontinuous gradient gels (precast-gel, BioRad Laboratories, Hercules, CA, USA), and run at 50 V for 20 min., followed by 80 V for 120 min, using a mini-gel apparatus (Mini-PROTEAN II Electrophoresis System, BioRad Laboratories, Hercules, CA, USA). Gels were stained with Coomassie® G-250 (SimpleBlue™ Safe Stain, Invitrogen Life Technologies, Paisles, UK) for 1 hr and destained with deionized water. Prestained molecular weight standards (see above) were run with each set of samples. As an internal MUP standard, we used 1 µl natural urine (i.e., not precipitated with acetone before SDS-PAGE) from a male mouse, that was loaded into 10 µl of sample buffer. Images were collected with a GS-800 Calibrated Densitometer (BioRad Laboratories, Hercules, CA, USA). Quantity One 1D Analysis Software (BioRad Laboratories, Hercules, CA, USA) was used for the densitometry analysis. The intensity of the MUP band from the mouse, relative to protein bands from the subterranean rodents, was calculated by the average optical density (OD) of MUP divided by average OD of all protein bands. The average OD of MUP also was divided by the average OD of the ~22.5 kDa protein bands of the subterranean rodents.

Urine from all rodents was tested by SDS-PAGE to assess the presence of proteins in the mass range of MUPs (or lipocalins) (Table 1). Samples from animals with such proteins were analyzed further by 2D gel electrophoresis.

2D Gel Electrophoresis - IEF (Isoelectric Focusing) Isoelectric focusing was performed on the urine specimens with the most concentrated bands in SDS PAGE, using the BioRad Protean® IEF Cell. Because the amount of protein in the samples was low, it was necessary to concentrate the urine before analysis. First, 800 µl of urine were centrifuged at 15,000 g for 10 min. Urine samples were fractionated in 300–400 µl aliquots, which were transferred into a Millipore-column (5 kDa, Ultrafree-MC) and spun at 15,000 g for 20 min. Samples were washed and centrifuged again at the same speed for 20 min., several times with a 1:4 times diluted PBS-buffer, until the stock solution was reduced to 50–100 µl. From this stock solution, 15 µl were precipitated in 250 µl acetone. Then, 7 cm IPG-strips (pH range 3.9–5.1 and 3–10, BioRad Laboratories, Hercules, CA, USA) were dissolved in 120 µl rehydration buffer (4.2 g urea, 1.5 g thiourea, 0.2 g CHAPS, 200 µl of 1 M

DTT, 10 μl of 0.5% bromphenol blue, 1 ml of 100x ampholytes pH 3–10; Bio-Lyte, BioRad Laboratoires, Hercules, CA, USA, in 10 ml distilled water). The strips were allowed to rehydrate passively overnight at 4°C. The program for focusing was as follows: 1 hr at 50 V, 25 μA per strip at 15°C, and a voltage limit of 4,000 V. Focusing was finished after 16,000 Vhr. Equilibration was done with 4 ml of two SDS equilibration buffers for 15 min. The first buffer contained 6 M urea, 0.375 M Tris pH 8.8, 2% SDS, 20% gycerol, 2.5% DDT. The second had the same components, but DDT was replaced by 2.5% iodoacetamide. The IPG strip was placed onto a 2nd dimension SDS-PAGE. An 8 μl sample molecular weight standard (Sigma-7-Ladder, BioRad Laboratories, Hercules, CA, USA) ran simultaneously with each set of samples.

MALDI–TOF-MS/MS (Matrix-Assisted-Laser-Desorption-Ionization – Time-of-Flight – Tandem-Mass-Spectrometry) For mass spectrometric analysis, proteins were isolated from pooled urine samples and concentrated by acetone precipitation. From each sample, 200 μg of protein were resuspended in 200 μl IEF rehydration buffer and applied on IPG strip (11 cm, pH 3–10; BioRad Laboratories, Hercules, CA, USA). Second dimension was carried out, as described above, on pre-cast gels (BioRad Laboratories, Hercules, CA, USA) stained with Coomassie® G-250. The major spot, with a molecular weight around 20 kDa, was excised from the gel and subjected to trypsin digestion. The gel spot was washed twice with 100 μl of 50 mM ammonium bicarbonate buffer in 50% acetonitrile and allowed to dry. Five ng of trypsin (Promega), in 10 μl of 50 mM ammonium bicarbonate and 10 μl of 97% ^{18}O water, were added, and the samples were incubated overnight at 37°C. Tryptic peptides were extracted with 1% trifluoric acid in 30% acetonitrile. A drop of extract was placed on the MALDI target plate and allowed to dry, and then covered with 2 mg/ml of α-cyano-hydroxycinnamic acid solution in 80% acetonitrile.

Tryptic peptides for *de novo* analysis were separated on PepMap 100 C18 RP column (3 μm particle size, 15 cm long, 75 μm internal diam; Dionex) with a gradient of 5% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid to 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, using an Ultimate 3000 HPLC system coupled to a Probot microfraction collector (Dionex). Matrix solution (2 mg/ml α-cyano-hydroxycinnamic acid in 80% acetonitrile) was added in the ratio 1:3.

MS spectra were obtained using a 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems/MDS Sciex) equipped with a Nd: YAG laser (355 nm, firing rate 200 Hz). MS/MS was performed with 1 kV collision energy and an operating pressure in the collision cell of 10^{-6} Torr. Spectra were interpreted manually using double peaks formed by $^{16}O/^{18}O$ to distinguish y-ion series. The series of b-ions was matched to corresponding y-ion series by subtraction of y-ion mass from parent ion mass.

Statistical Analyses We used the *Kruskal Wallis one-way ANOVA* for independent samples to compare mean total urinary protein concentrations (Bradford test) across animals of different genera, because the data were not normally distributed (*Kolmogorov-Smirnov test*). The *Mann-Whitney U-test* for independent samples was used to compare mean total urinary protein concentrations (Bradford test) for males and females. All statistical analyses were performed with SPSS 15. Means are given as $\bar{x} \pm SD$.

Results

Bradford Test The mean total urinary protein concentration for the experimental animals was 0.45±1.09 SD mg/mg Cr, with no differences (except for mice) between males and females [tested separately for each genus: *Mann-Whitney U-test* (*Fukomys*: U=132.0, P=0.90; *Spalax*: U=31.0, P=0.23, *Spalacopus*: U=10.0, P=0.37, mice: U=7.0, P< 0.01)], and with comparable values at the genus level (*Kruskal Wallis one-way ANOVA*, χ^2=3.168, P=0.205) (Fig. 1). The urinary protein concentration of mice (12.4± 4.4 SD mg/mg Cr) was higher than that of the subterranean species (*Kruskal Wallis one-way ANOVA*, χ^2=49.62, P< 0.001) (Fig. 1). In contrast to the generally low values found for the subterranean rodents, there were three

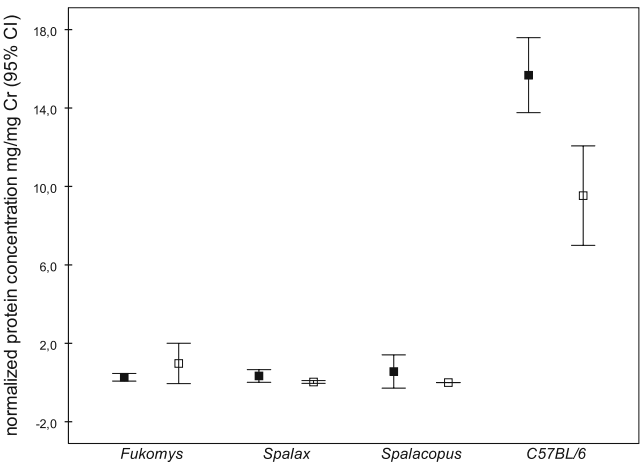


Fig. 1 Normalized total urinary protein concentration (mg/mg Cr) of both sexes of *Fukomys* (N=17 males, 16 females), *Spalax* (N=16 males, 6 females), *Spalacopus* (N=8 males, 4 females), and C57BL/6-mice (N=9 males, 10 females). Filled squares represent means of males, open squares females, with error bars signifying confidence intervals. Cr=creatinine

reproductively active *Fukomys* females (queens) with high total protein concentrations (individual #3: 2.7 mg/mg Cr, individual #12: 2.4 mg/mg Cr, and individual #17: 7.3 mg/mg Cr). The higher concentrations in these samples also were conspicuous in SDS-PAGE (Fig. 2).

SDS-PAGE In the qualitative SDS-PAGE analysis of urine samples of the three subterranean genera under study, only *Fukomys* showed one or two consistent bands (approximately ~22.5 kDa) (Fig. 2). In *Spalax*, only the samples of two males (out of 25 animals) had one band of approximately ~19–20 kDa (Fig. 3). None of the *Spalacopus* specimens revealed a band in the MUPs or lipocalin mass range (cf. Table 1).

Given that all *Fukomys* individuals showed a band in the MUPs mass range, we analyzed their SDS-PAGE pattern in order to investigate polymorphisms related to species, sex, or reproductive status (e.g., queens vs. non-reproductive daughters). No protein mass polymorphisms could be detected for *Fukomys* by this method. Band patterns, in the range of 22–23 kDa, consisted mainly of two bands (23 out of 31 samples); all other animals showed a pattern of one band (8 out of 31) (Fig. 2).

2D Gel Electrophoresis The most concentrated *Fukomys* samples came from two males (numbers 11 and 14), and these, along with those of two females (numbers 4 and 22; Fig. 2), were used for 2D gel electrophoresis. The only samples from *Spalax* that contained detectable amounts of protein were samples 5 and 7 (Fig. 3). A sample from a male mouse was used as a reference. The short range 2D PAGE (isoelectric point: pI=3.9–5.1) showed similarity only between spot 1 of the mouse and spots 5 and 6 of *Fukomys* (#11 m). They differed slightly (about 3 kDa) in mass, and the pI also was different (Fig. 4). None of the spots could be detected in the three other *Fukomys* specimens (e.g., #1 m, Fig. 4).

In the broad-range 2D PAGE (pI=3–10), two spots (spot 1 and 2) of the tested *Fukomys* specimen (#11 m) were nearly congruent to spots 1 and 2 of the mouse, although they differed slightly in both parameters: mass by approximately 1–3 kDa and pI (broad range not shown here, c.f., Fig. 4). The other two spots of the mouse did not correlate with the spots found in *Fukomys* urine samples.

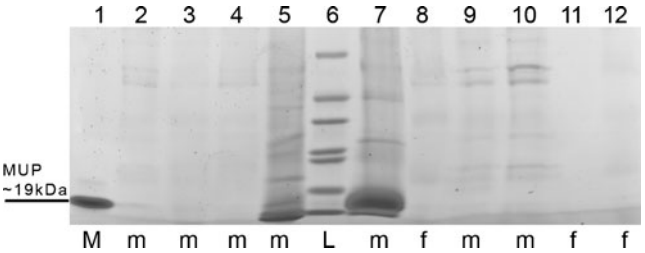


Fig. 3 SDS-PAGE of urine of males (m) and females (f) of *Spalax ehrenbergi* species complex. Sigma-7-ladder (L) and C57BL/6-mouse (M) as internal (qualitative) standard. Where major urinary proteins (MUPs) are expected is given

Of the two tested *Spalax* individuals, spot 3 of animal #7 m had the same range in both parameters as the *Fukomys* and mouse specimens (Fig. 5). None of the other numerous spots of this individual, nor any of the spots of individual #5, exhibited similar characteristics to the spots of the mouse or those of *Fukomys*.

A trend emerged in *Fukomys*: spot 1 was approximately similar to the mouse, although slightly different in mass (20–21 kDa) and pI; spot 2 existed in two male *Fukomys* individuals (#11 and #14); spot 3 in male #11, #14, and one queen (#4); the characteristics of spot 4 for males #11 and #14 were similar to the mouse. In sum, those three spots may be typical for *Fukomys* mole-rats. Both spots that, more or less, fit the pattern of the mouse were detected in just one out of four *Fukomys* specimens.

MALDI–TOF-MS/MS Peptide mass fingerprints were matched against a sequence database to identify the proteins present in the 20 kDa spot of the 2D-PAGE gel of *Fukomys*. No significant hits were obtained based on peptide masses. Subsequent *de novo* sequencing revealed the sequence of the peptide, which showed high similarity to the sequence of hamster aphrodisin (GI:1168469, BLAST, Basic Local Alignment Tool, www.ncbi.nlm.nih.gov/blast). The fragment differs from the corresponding aphrodisin sequence by four amino acid residues: YJAADNVEKJEEGGELR (where J is Leu or Ile, and K is Lys or Gln residue). Changes are indicated in grey.

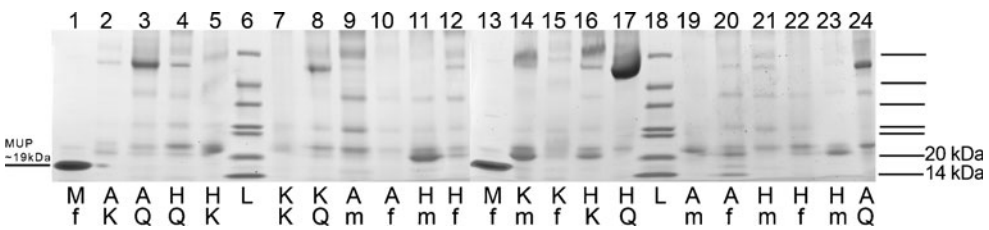


Fig. 2 SDS-PAGE (precast 4–15%) of urine from non-reproductive males (m), non-reproductive females (f), reproductive males (K, kings) and reproductive females (Q, queens) of *Fukomys anselli* (A), *F. kafuensis* (K) and their hybrids (H). Migration from top to bottom. Sigma-7-ladder (L) and C57BL/6-mouse (M) as internal (qualitative) standard. Where major urinary proteins (MUPs) are expected is given

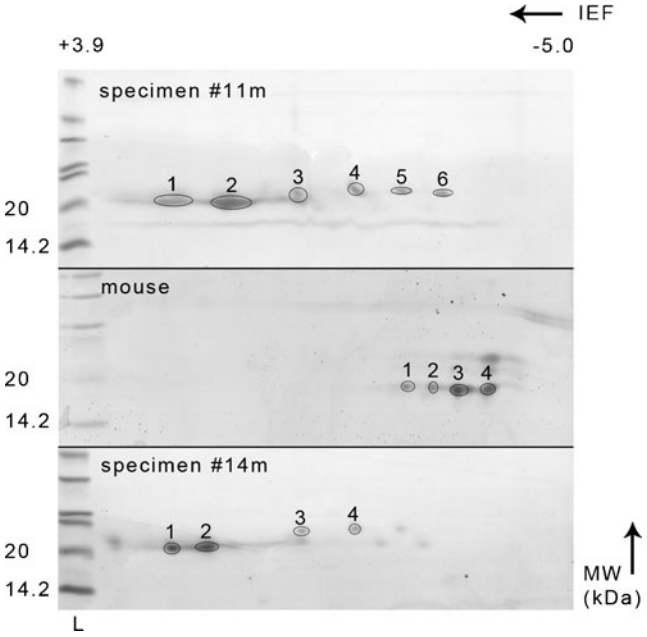


Fig. 4 2D PAGE of urine from two male *Fukomys* individuals and a mouse. Isoelectric focusing (IEF) was conducted with low range strips, with the isoelectric point (pI) ranging from 3.9 to 5.1. Sigma-7-ladder (L). The specimen number refers to the lane number in Fig. 2

Discussion

The biochemical analyses indicated the absence of MUPs in urine of the three tested rodent genera. No urinary protein in the mass range of ~20 kDa could be detected in *S. cyanus*. Of the 25 individuals tested from the four species of the *S. ehrenbergi* superspecies, one individual (#5) showed an SDS-PAGE band in the range of ~14 kDa, while another individual (#7) showed one band in the ~18 kDa range. These bands could be expanded through 2D gel electrophoresis to reveal two spots (~11 and ~14 kDa) in the first individual, and several spots, ranging from ~11 to ~20 kDa, in the second individual. High urinary protein excretion can be an indication of proteinuria (e.g., Rustom et al., 1998; Ghiggeri et al., 2004). Although there were no overt signs of illness at the time this individual's urine was collected, this mole rat may have been ill, particularly as 23 other *Spalax* samples showed no protein bands. In all the *Fukomys* samples, one or two bands in the mass-range of ~22–23 kDa were identified, but there was no indication of mass polymorphism according to species, sex, or reproductive status. 2D gel electrophoresis further demonstrated in one *Fukomys* male, two spots with similar charges to those of the mouse (pI 4.4–4.9 and pI 5.0–5.4), although they had a different mass range (~20 and ~22 kDa vs. ~19 and 19.5 kDa). The typical spot pattern in *Fukomys* consisted of three spots that were in the range of pI 4.05–4.57 with a mass of ~20–23 kDa.

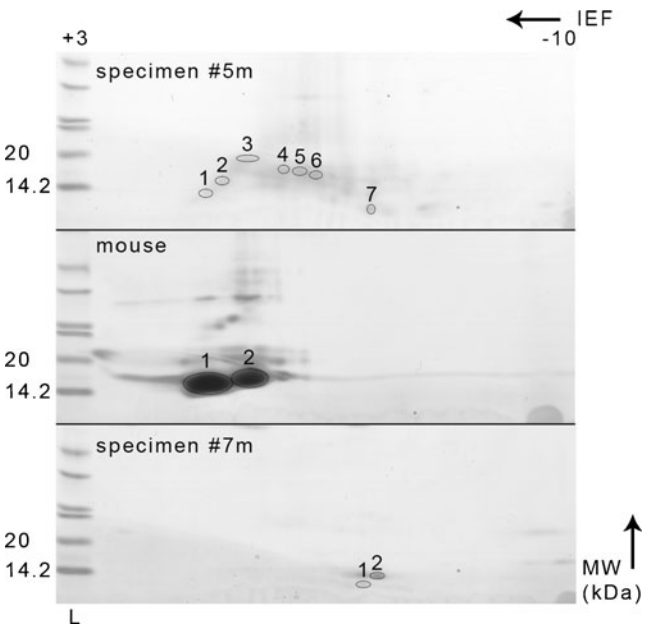


Fig. 5 2D PAGE of urine from two male *Spalax carmeli* and a mouse. Isoelectric focussing (IEF) was conducted with broad range strips, with the isoelectric point (pI) ranging from 3 to 10. Sigma-7-ladder (L). The specimen number refers to the lane number in Fig. 3

Although the urinary proteins of *Fukomys* were somewhat similar to the mouse in mass and charge, Bradford analysis revealed very low mean total urinary protein concentrations in *Fukomys*; protein concentrations were 19 times lower in *Fukomys* than in the mouse, and 27 times lower when all analyzed urine samples were considered. Although subterranean rodents do not drink free water, they consume enough water from their food to produce large amounts of urine, along with substantial calcium and magnesium carbonates, suggested to be a means of reducing CO_2 in the hypercapnic subterranean environment (Haim et al., 1985). This might explain the low protein concentrations. Total urinary protein concentrations were sufficiently low in both sexes of the subterranean species, in comparison with the mouse, so it is unlikely that urinary proteins play more than a trivial role, if any, in chemical communication in these species.

According to the average OD, the MUP band of the mouse is substantially more concentrated than all protein bands of *Fukomys*. The discrepancy, between this and the Bradford measures of total urine protein (see above), may be because proteins respond differently in OD measurements. Nevertheless, both types of data (Bradford and SDS-PAGE) demonstrate substantial differences in the amount of urinary proteins in the mouse compared to *Fukomys*. Whereas, the urinary proteins of mice are 99% MUPs, only one quarter of *Fukomys* urinary proteins is represented by the ~22.5 kDa band (mean ratio of the average OD all bands/~22.5 kDa). MALDI-TOF analysis identified the ~22–23 kDa band of *Fukomys* as comparable to aphrodisin, rather than a MUP-type lipocalin, confirming that none of

the subterranean rodent genera investigated, produce MUPs in their urine.

Interpreting these unexpected findings in the context of the behavioral evidence about how these subterranean rodents respond to urine odors, as well as in the context of findings about MUPs in other rodent species, could provide insight into a broad understanding of the processes underlying transmission of semiochemicals in rodents. All the rodent species analyzed here discriminate between individuals on the basis of odors in urine or urogenital and/or ventral secretions that may also include some urine (in *Spalax*, Todrank and Heth, 1996; Heth and Todrank, 2000; in *Fukomys*, Heth et al., 2004; and in *Spalacopus cyanus*, Hagemeyer and Begall, 2006). Studies, published while our study was underway, also noted that MUPs could not convey individual identity in species in which there was no polymorphism in MUPs, as in *Mus macedonicus* (Robertson et al., 2007), nor in species in which the patterns of urinary lipocalins across individuals were "remarkably consistent", as in *M. spretus*, as well as wild-caught rats, *Rattus norvegicus*, and the Roborovski hamster, *Phodopus roborovskii* (Beynon et al., 2008).

In species lacking polymorphism in MUPs, it is still possible to posit a role for MUPs in transmitting information about reproductive readiness or dominance status by variations in the concentration of MUPs (Beynon et al., 2008). However, in species lacking MUPs altogether, like the rodent species in the present study, there must be another mechanism for disseminating information about status and identity. This must be true for any rodent species, irrespective of ecotope, such as the wood mouse, *Apodemus sylvaticus* (family Muridae), which lacks urinary proteins in the 18–20 kDa range typical of lipocalins (Stopka, unpublished data). Although the behavioral research on the species in this study focused primarily on aspects of individual identity and responses to genetic similarity/difference, *Fukomys* and *Spalax* both responded to distinctions in urine that are indicative, for example, of reproductive state (Heth et al., 1996; Hagemeyer, 2010). It is clear that communication of information about reproductive status is accomplished in *Spalax*, despite their lack of both MUPs and aphrodisin. Additional research would be necessary to determine what role, if any, the trace amounts of aphrodisin found in *Fukomys* urine serves. Demonstrating a role for the aphrodisin-like odor-binding proteins found in bank voles (Stopková et al., 2010) would also contribute to a more thorough understanding of the extent to which urinary proteins influence chemical communication in rodents.

Chemical communication of both individual identity and biological state is so essential in the life of rodents that the fundamental mechanisms of chemosignaling have probably been conserved, despite adaptation to different ecotopes and social structures. Because rodent species lacking MUPs or polymorphic MUPs respond adaptively to chemosignals conveying information about individuality, species, and biological state, the basic process underlying transmission of this information cannot rely universally on MUPs. Indeed, a recent study characterizing MUP genes and pseudo-genes in a wide array of placental mammals, from rodents to horses, found extensive MUP gene polymorphism only in *M. musculus*, and moderate polymorphism only in *Rattus norvegicus* (Logan et al., 2008), suggesting that the separate expansion of the MUP gene cluster in *Mus* and *Rattus* may be atypical among mammals. It is, therefore, conceivable that the use of MUPs in mice with polymorphic MUPs is a secondary process that augments the primary process of conveying identity or status directly, perhaps by extending the availability of the semiochemicals in urine. If there is a universal process that transmits semiochemicals from sender to receiver across Rodentia, then the search for such a process must continue, because MUPs are not sufficiently prevalent to accomplish this task.

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Polar Metabolites Synergize the Activity of Prostaglandin $F_{2\alpha}$ in a Species-Specific Hormonal Sex Pheromone Released by Ovulated Common Carp

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Abstract Many species of teleost fish detect and release F prostaglandins (PGFs), but the specific identities of these compounds and how they function as species-specific pheromones have yet to be resolved. This study addressed these questions in the common carp. An initial set of experiments established that mature male common carp were attracted to chemicals released by ovulated conspecifics, whereas the odor of female goldfish, a close relative, was less attractive. Tests of fractionated holding water from ovulated carp revealed that only the non-polar fraction was attractive on its own. Mass spectrometry and immunoassay next demonstrated that the non-polar fraction contained large quantities of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 15keto-prostaglandin$F_{2\alpha}$, and 13,14-dihydro-15keto-prostaglandin $F_{2\alpha}$ (100 g fish released over 1 μg of all 3 PGFs per h at a ratio of 1.0: 1.7: 0.7). Ovulated goldfish released the same three PGFs but at a slightly greater rate and in a different ratio. Tests of synthetic mixtures of these PGFs revealed that the carp-specific mixture attracted male carp but was no better than the goldfish-specific mixture or $PGF_{2\alpha}$ alone and that $PGF_{2\alpha}$ was just as attractive as mixture of all three PGFs. A final set of attraction tests revealed that although $PGF_{2\alpha}$ could explain all of the activity of the non-polar portion of female carp holding water, it could not explain the entire activity of female water but that a mixture of PGFs and the polar fraction could. We conclude that ovulated female carp release a multi-component sex pheromone complex that is comprised of $PGF_{2\alpha}$ and unknown species-specific polar compound(s) that synergize the activity of the former. The pheromone also might be useful in controlling this invasive species. The observation that a fish hormonal sex pheromone incorporates bodily metabolites in addition to relatively common hormonal products demonstrates a mechanism by which species specificity may be conferred to this common type of sex pheromone.

Key Words Common carp · *Cyprinus carpio* · Electrospray ion mass spectrometry (ESI-MS/MS) · Enzyme-linked immunosorbent assay (ELISA) · Goldfish · Hormonal pheromone · Invasive species · Mass spectrometry (MS) · Pheromone complex · Species-identifying pheromone

Introduction

Fish rely on sex pheromones to mediate various aspects of their reproductive biology, and many of these appear to be at least partially comprised of hormones and their derivatives and are known as 'hormonal pheromones' (Stacey and Sorensen, 2009). Sex pheromones released by recently-ovulated females are some of the best understood hormonal pheromones. They often appear to contain F prostaglandins (PGFs), which also are known to serve as endocrine mediators of ovulation and female spawning behavior (Sorensen and Goetz, 1993; Stacey and Sorensen, 2009). The seemingly common use of PGFs as pheromones is supported by much physiological and behavioral evidence, but proof of their precise identities and function within ovulated female odor remains to be elucidated. Thus, while approximately a dozen electrophysiological studies of olfactory function in a variety of fishes have shown that they detect prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and two of its metabolites with acute sensitivity and specificity, evidence

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of PGF release comes from only a few species and relies entirely upon either immunoassays (whose interpretation can be complicated by antibody specificity), or HPLC (whose interpretation can be complicated by co-elution of related structures) (Sorensen and Goetz, 1993; Sorensen et al., 1995; Stacey and Sorensen, 2009). Further, while laboratory studies have consistently shown PGFs to stimulate arousal and /or attraction in males of those species that detect them (see Stacey and Sorensen, 2009), responses have not yet been compared to those elicited by ovulated female odor to see if they are identical. Thus, the specific identities, amounts, and functions of PGFs released by ovulated fishes are not established.

Because the identity of pheromonal PGFs is unresolved, questions about whether and how they might account for pheromone specificity also persist. Complicating this situation is the fact that it is not yet clear whether the odors (pheromones) of ovulated females are always species-specific or why. Thus, while approximately a dozen species of fish have been shown to release odors with species-specific actions when ovulated (Sorensen and Stacey, 1999), a few species appear to release cue(s) that are not species-specific (ex. McLennan and Ryan, 1997) (hence, our usage of the seemingly redundant term "species-specific pheromone"). The precise significance of these observations is difficult to ascertain because no study has yet attempted to link directly the release of any particular compounds including PGFs to pheromonal specificity to test whether/ how they might explain either the full activity or the apparent specificity of the odor of ovulated fish. Curiously, only three PGFs ($PGF_{2\alpha}$, 15keto-prostaglandin $F_{2\alpha}$ [15K-$PGF_{2\alpha}$], and 13,14-dihydro-15keto-prostaglandin $F_{2\alpha}$ [dh15K-$PGF_{2\alpha}$]) have been shown to have pheromonal function to date (see Sorensen and Stacey, 1999; Stacey and Sorensen, 2009).

There are at least four, non-mutually exclusive ways that these seemingly common hormonal products might explain the apparent species-specificity of ovulated pheromones. One possibility is that female fish release and male fish detect highly prescribed and distinctive mixtures of these PGFs (Sorensen et al., 1998). Although the finding that goldfish, *Carassius auratus,* possess multiple olfactory receptors for PGFs adds credence to this hypothesis (Sorensen et al., 1988), its relevance to pheromonal specificity has not been tested. Another possibility is that some female fish may release novel PGFs that have not yet been discovered. A third possibility is that PGFs do not themselves convey species information but rather this information is conveyed by metabolic compounds released along with them. The finding that juvenile fish of a variety of species discern species identity by using an odor that includes both polar and non-polar components (Sisler and Sorensen, 2008) supports this possibility (Levesque et al., 2011), but it has not been tested in mature, sexually-active adults. Fourth, it is possible that hormonal pheromones themselves are not always species-specific, and that this information is instead conveyed by contextual information such as visual appearance and temporal patterning of pheromone release (Sorensen and Stacey, 1999). The finding that female goldfish release PGFs in highly regulated urinary pulses supports this possibility (Appelt and Sorensen, 2007). Direct tests of the potency of ovulated female odor and the various components it contains are lacking for any fish and now are needed to distinguish among these possibilities.

The common carp, *Cyprinus carpio,* (hereafter 'carp'), is a good model to test these questions. It is a close relative of the goldfish and is one of the only species for which detailed information exists on prostaglandin release, detection, and response (Sorensen and Goetz, 1993; Kobayashi et al., 2002). To date, lessons learned from the goldfish have proven applicable to many fishes, although distressingly few species have been studied, and few exhibit radically different reproductive strategies (Stacey and Sorensen, 2009). Interestingly, although the goldfish and carp have strikingly similar behaviors, endocrine systems, and olfactory sensitivities to PGFs (Irvine and Sorensen, 1993; Sorensen and Stacey, 2004), they do not commonly hybridize, suggesting they employ some type of species isolating mechanism (Sorensen et al., 1992; Hänfling et al., 2005). Because both male goldfish and carp suffer from severe reductions in spawning activity if their olfactory systems are occluded (Stacey and Kyle, 1983; Lim and Sorensen, unpublished results), it is likely that female pheromones are involved with this process. The carp is an invasive species (Weber and Brown, 2009) and complete identification of its sex pheromones would be useful in its detection and control (Sorensen and Stacey, 2004).

The overarching goal of the present study was to investigate whether the carp uses a $PGF_{2\alpha}$-based female-derived sex pheromone to encode species specificity at the time of ovulation and spawning. Four questions were addressed: (1) Do ovulated female common carp release a sex pheromone? (2) If so, is this pheromone species specific? (3) Do PGFs play a dominant and/or exclusive role in this pheromone? and (4) Are the ratios of PGFs released by different species of fishes important to pheromone function?

Methods and Materials

Study Design The study investigated the role of PGFs in the female carp pheromone in four sets of experiments whose designs were based on each other's findings. Experiments employed an attraction assay that used circular

mazes that we have used before (see section below). Each experiment tested a single treatment (odor), and the results of related sets of experiments were then compared to each other and a control to answer questions about the identity and role of PGFs. Several follow-up experiments tested odors of special interest against each other in 'head-to-head' tests to determine their relative potencies. Odor fractionation/purification employed reverse-phase C18 resin, which is known to separate aqueous polar and non-polar structures (see Levesque et al., 2011) while both mass spectrometry/mass spectrometry (MS/MS) and enzyme-linked immunosorbent assays (ELISA) were used to analyze for the presence of PGFs in both carp and goldfish waters. This combination of techniques permitted cross-validation.

Question 1. Do Ovulated Carp Release a Sex Pheromone that Attracts Males? First, we determined whether ovulated carp release a sex pheromone by testing the behavioral responses of male carp to holding water from ovulated carp, holding water from non-ovulated female carp, and blank well water (control). The holding waters for these experiments were created by placing ≈200 g of ovulated or non-ovulated fish (typically 3 fish) into plastic containers containing 10-l of aerated 20°C well water for 1 h following established protocols (Sisler and Sorensen, 2008; Levesque et al., 2011). Matching controls for each experiment were created in the same manner without fish. Ovulation was induced by injecting females with hormones (0.5 ml Ovaprim/kg body weight, Syndel Inc., Vancouver, Canada) 12 h in advance of when they were needed (typically 2000 h). Ovulated fish were collected the next morning [0800 h; approximately 4 h after ovulation and at a time that steroidal pre-ovulatory pheromone levels would have been very low (Scott and Sorensen, 1994)], rinsed in well water to further minimize any possibility of including pre-ovulatory pheromones (Stacey and Sorensen, 2009), and then incubated. Control females were treated in the same manner except they were injected with saline and thus did not ovulate. All fish were handled carefully using well-rinsed nets and placed into clean containers with well water for holding water collection.

Question 2. Is the Pheromone Species-Specific? Having established the ovulated carp release an attractant, we tested the specificity of this attractant by describing the behavioral responses of male carp to ovulated carp holding water, ovulated goldfish holding water, and then to a choice of the two. A well water control was also tested. Responses of male goldfish to a choice of carp and goldfish waters were evaluated in a fifth experiment. Protocols for odor collection and testing followed those used to answer question 1 except that water samples were collected in advance and frozen until needed when they were thawed in a 20°C water bath. Pilot tests established that freezing/thawing did not affect the attractiveness of ovulated carp water [male carp offered a choice of fresh and previously frozen ovulated carp water did not distinguish between them (relative attraction value was 1.8±0.7%; N=3)].

Question 3. Do Known PGFs Play a Dominant Role in the Female Odor (the Entire Pheromone)? This question was addressed in a step-wise fashion by several subsets of experiments that addressed the importance of the non-polar fraction (which we already knew to contain all PGFs (Eom et al., 2009; Sorensen, unpublished data), the identity of the PGFs found in the non-polar fraction, the relative importance of the identified PGFs in this fraction, and finally the importance of PGFs in the entire odor. For the first subset of experiments, 1-l samples of ovulated carp holding water or well water control were passed through activated reverse phase C18 columns (Sep-Pak, Waters Inc, Milford, MA, USA), eluted with 5 ml of methanol (Sigma-Aldrich, St. Louis, MO, USA), dried under a stream of nitrogen, reconstituted in 100 µl of methanol, and dissolved in 1-l of well water for testing. Meanwhile, the filtrate (i.e., the polar fraction) was collected and tested directly. Ovulated carp holding water and a blank well water control also were tested on their own as controls. Pilot studies confirmed that treating water with C18 columns had no effect on carp behavior (relative attraction value was 0.8±0.4%; N=2). For the second subset of experiments, we analyzed the PGF content of C18 extracts of 5 ovulated carp and 3 ovulated goldfish holding waters by using both MS/MS and ELISA (see section below) on all samples. Next, we tested the importance of these PGFs in the non-polar fraction by creating a carp-specific mixture of PGFs using synthesized $PGF_{2\alpha}$, 15K-$PGF_{2\alpha}$, dh15K-$PGF_{2\alpha}$ (purity >98%; Cayman Chemical, Ann Arbor, MI, USA), and then tested this mixture on its own, and then against the non-polar fraction from ovulated carp water. Awell water control was also tested as part of this subset of experiments. Lastly, in a fourth subset of experiments, we determined the importance of these PGFs within the entire pheromone by testing the mixture of $PGF_{2\alpha}$, 15K-$PGF_{2\alpha}$, and dh15K-$PGF_{2\alpha}$ against thawed ovulated carp holding water, and then adding it to filtrate and testing this complete mixture against ovulated water.

Question 4. Is the Specific Ratio of PGFs Found in Female Holding Water (the 'Entire' Pheromone) Important to its Activity? Although the previous set of experiments found PGFs to be important, they did not address the possible role that mixtures of PGFs might play in specificity. This was addressed by three additional subsets of experiments. First, we tested the activity of each PGF by testing each at a concentration of 2×10^{-10} Molar (M), the lowest concentration measured in holding water, and then at 6×10^{-10} M, a concentration equal to the sum of all three

PGFs. Control experiments that tested well water control were included in each of these sets of experiments. Second, to test whether the PGF ratio might convey species-specific information, we evaluated the mixture of the PGFs found in ovulated carp water against a mixture of the PGFs found in ovulated goldfish water in a head-to-head test. A final experiment assayed the carp-specific mixture of PGFs (6×10^{-10} M) and against $PGF_{2\alpha}$ (2×10^{-10} M), the only component found to be active on its own.

Experimental Fish Carp and comet variety goldfish were purchased from fish farms (Osage Catfisheries, MO, USA and Hunting Creek Fisheries, PA, USA, respectively) and shipped to the university where males and females were held into different 1000-l flow-through circular tanks supplied with 20°C well water at a rate of 10-l/min. All fish were fed a mixture of flake fish food (Aquatic Eco-Systems, Apopka, FL, USA) and frozen brine shrimp (San Francisco Bay Brand, Newark, CA, USA) every day while being maintained on a 16:8 h (L:D) photoperiod. The goldfish were sexually mature when they arrived, but the common carp had to be raised to maturity (this took 1 yr) and sorted by sex after they matured (based on body shape and sperm production). The day prior to testing, male fish were 'primed' (placed into a state of moderate sexual arousal) by placing them into 100-l tanks that contained 10^{-10} M 17α,20β-dihydroxy-4-pregnen-3-one (Sigma-Aldrich, St. Louis, MO, USA), the major component of the pre-ovulatory steroidal pheromone. Previous studies have shown that this pheromone greatly enhances behavioral responsiveness to the odor of prostaglandins (deFraipont and Sorensen, 1993; Lim and Sorensen, unpublished results). Males were selected randomly for all experiments and re-used up to 3 times after being given at least a month to recover in separate holding tanks to minimize any chance that previous experience might affect results. At the time of testing, male common carp were spermiated (i.e., they released sperm when squeezed), had an average weight of 89.7±10.3 g, and an approximate gonadosomatic index (GSI =gonad weight/whole body weight × 100%) of 6.3±0.8% (mean ± standard error). Female carp had mature eggs, weighed 100.5±11.9 g, and had an approximate GSI of 11.5 ±2.1%. Female goldfish had an average weight of 41.3± 7.1 g and GSI of 12.8±3.4%. Gonadosomatic indices were determined by euthanizing 4 fish at the conclusion of experiments using 0.1% MS222 following the institutional animal use and care protocol.

The Attraction Assay The attractiveness of test odors to male carp was evaluated by using a protocol based on one that we have used before (Maniak et al., 2000; Rolen et al., 2003; Sisler and Sorensen, 2008; Levesque et al., 2011). In this assay, the distribution of groups of fish is observed in darkened circular mazes, each of which has two arms and a central, drained, neutral area (Fig. 1). Test and control odors are added to one side arm and/or the other arm following a pre-test period. We use groups because carp are social and behave more naturally when tested in this manner. For this study, each experiment was repeated 10 times using different groups of male fish, each of which was considered an independent trial. Trials commenced by placing groups of 5 primed males into a circular maze tank after which incoming water was turned off and they were allowed to acclimate for 1 h. This was then followed by a 15-min pre-test period during which time well water control was added to both test areas of the maze, after which an odor was pumped into the one test area while well water control (or in the case of head-to-head tests, another odor) was pumped into the other side for another 15-min for the test-period. Test odors were added to the test area with the fewest carp during the pre-test, and were introduced (10 ml/min) via small plastic tubes connected to air-stones which promoted mixing. Dye tests demonstrated that odors were diluted about 10 fold within a distance of 10 cm from the point of introduction, and did not cross between test areas for 15-min. Mazes were covered by black curtains and illuminated by two overhead infrared lights (Vitek, Taipei, Taiwan) and dim (5W) incandescent lights while low light cameras (Bosch Security Systems, Germany) were positioned overhead and connected to a monitor and a DVD recorder.

Each trial was analyzed independently by reviewing DVDs. To accomplish this, the number of carp observed in the test area of the maze was counted at 15-sec intervals during the pre-test period and then the test period (the individual identities of fish were not noted). The numbers

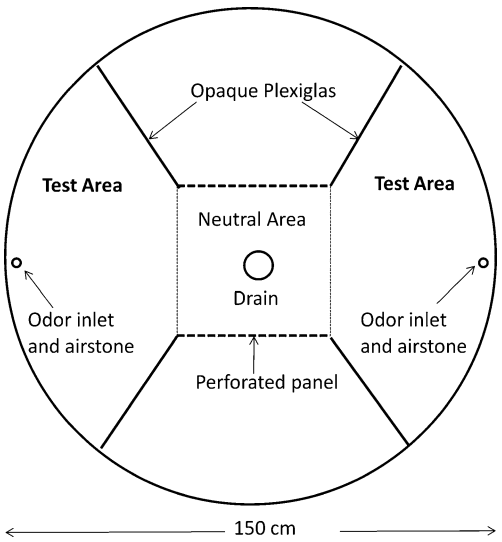


Fig. 1 A schematic drawing of the two-choice maze used to test behavioral responses of primed male common carp, *Cyprinus carpio*. Fine dashed lines mark the borders between test and neutral areas. The maze was filled to a depth of 20cm

of fish seen were summed for each period, and divided by 300 [the total possible score (i.e., 5 fish × 60 15-sec time periods)] to yield percent time that all fish spent in the experimental side during each period. Pre-test values were subtracted from test values for each trial to yield 'relative attraction', a measure of change in time spent in the test area during odor addition. The use of relative attraction served to normalize the data and remove any possible side bias. An average value for relative attraction next was calculated for all 10 trials of each experiment and differences between treatments (test odors) compared by using one-way ANOVAs (SigmaStat, Ashburn, VA, USA). If significance was indicated ($P<0.05$, $P<0.01$, or $P<0.001$), individual values were compared to each other and the blank control using a Holm-Sidek adjustment (Aickin and Gensler, 1996). In addition to these analyses, values for blank control also were tested against zero using *t*-tests to confirm a lack of maze bias. Head-to-head experiments were evaluated independently by comparing relative attraction values with *t*-tests (SigmaStat, Ashburn, VA, USA) after confirming normality with a Kolmogorov-Smirnov test (Sigmastat).

Biochemical Analysis of PGFs Goldfish and carp samples were analyzed by using a combination of electrospray negative ion mass spectrometry (ESI-MS/MS) and ELISA. We used both techniques on both species because neither technique could be fully definitive on its own. In particular, while the immunoassays we employed were highly specific, the possibility that as yet unidentified PGFs might be interfering with measurements could not be ruled out. Additionally, ESI-MS/MS cannot differentiate between $PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$ as they have identical molecular weights and fragment in similar manners. Thus, we used both techniques to cross-check each other's findings.

For ESI-MS/MS, 5 µl aliquots of C18 extracts of carp and goldfish holding waters were injected into an ion trap mass spectrometer (LCQ-1, ThermoScientific, Waltham, MA, USA) equipped with an electrospray ionization source [ion trap was operated in the negative ion mode with a spray voltage of 5 kV; sheath gas was 99% pure nitrogen at 60 psi; sheath fluid was 50:50 20 mM triethylamine: acetonitrile (v/v)]. Ionization patterns were compared with standards of $PGF_{2\alpha}$, 15K-$PGF_{2\alpha}$, and dh15K-$PGF_{2\alpha}$, the three PGFs previously described in ovulated fish extracts (Sorensen et al., 1988, unpublished). For quantification, standards (Cayman Chemical, Ann Arbor, MI, USA) were made up in blank holding water at concentrations ranging from 3.5–175.5 ng/l and then extracted with activated C18 columns to produce a calibration curve that corrected for possible matrix effects and extraction efficiencies (Fine et al., 2006). Daughter ions of *m/z* 291, 309, 317, and 335 were monitored to quantify $PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$, and *m/z* 315 and 333 for 15K-$PGF_{2\alpha}$.

To analyze by immunoassay we used duplicate samples and commercially available kits for $PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$ (15K-$PGF_{2\alpha}$ was not re-analyzed because MS/ MS was deemed to give distinctive reliable measurements). For $PGF_{2\alpha}$, we used an antisera that reacted 100% with $PGF_{2\alpha}$, 90% with $PGF_{1\alpha}$, <4% with thromboxane B2 and 6-keto-$PGF_{1\alpha}$, and <1% with 2,3-dinor-6-keto-$PGF_{1\alpha}$, 11b-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, and 15K-$PGF_{2\alpha}$, dh15K-$PGF_{2\alpha}$ (Neogen, www.neogen.com, catalog #404710). To analyze for dh15K-$PGF_{2\alpha}$, we used an antisera that reacted 100% with dh15K-$PGF_{2\alpha}$, and less than 1% with $PGF_{2\alpha}$, 15-keto-dihydro-PGE_2, and 15K-$PGF_{2\alpha}$ (Neogen, www.neogen.com, catalog #404610). For each ELISA test, the eluate was dried down under nitrogen and re-suspended in the buffer supplied with the kits. Samples were then run in duplicate along with 8-point standards and blanks (0–1 ng/ml). Most samples had to be run several times after diluting them 10–1,000 times to produce values that fell on the 8-point calibration curve. Final ELISA results were confirmed by adding the values generated by the ELISAs for both $PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$ and then comparing them with the value generated by ESI-MS/MS by *t*-test.

Results

Analyses of pre-test data found neither a side bias in any of our mazes nor any effect of adding blank control ($P>0.05$; $N=25$ for all 4 sets of experiments). Our first set of experiments found that primed male carp were attracted to ovulated carp holding water ($P<0.001$ vs. control), but not to the well water control ($P>0.05$) nor to non-ovulated female carp holding water ($P>0.05$; Fig. 2a). Our second set of experiments confirmed the behavioral potency of ovulated water and showed that responses to this cue were species-specific; although ovulated goldfish holding water was attractive ($P<0.05$), paired comparisons showed it to be less so than ovulated carp water ($P<0.05$). Further, when the two stimuli were compared directly in head-to-head tests, the ovulated carp odor was preferred ($P<0.001$; Fig. 2b). Tests with male goldfish showed that they too preferred ovulated goldfish water to that of carp (mean relative attraction=14.5%±1.7; $P<0.001$; $N=10$).

Experiments designed to answer question 3 found that while the polar fraction (filtrate) was not attractive to male carp when tested against the control ($P>0.05$), the non-polar fraction (eluate) was ($P<0.001$) (Fig. 3). Analysis of the non-polar eluate of female carp and goldfish water by ESI-MS/MS found negative ions with *m/z* ratios of 291, 309, 317, and 335, which corresponded to both $PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$ (their profiles were identical) and *m/z* ratios of 315 and 333, which corresponded to 15K-$PGF_{2\alpha}$ (Fig. 4)

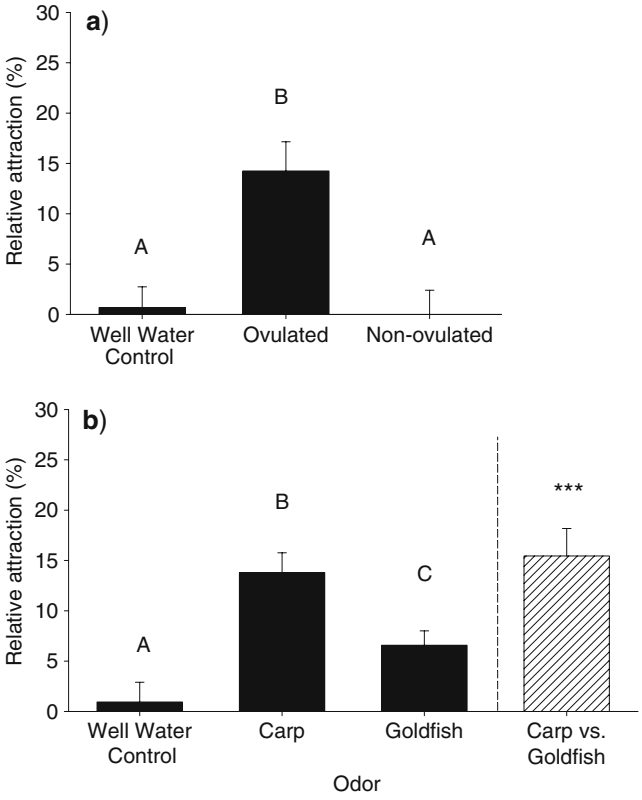


Fig. 2 Responses (mean relative attraction ± standard error) of primed male carp, *Cyprinus carpio*, to: **a** Blank well water control, holding waters of ovulated and non-ovulated female carp; **b** Blank control, holding water of ovulated carp, and holding water of ovulated goldfish, *Carassius auratus*. Stimuli were tested against appropriate matching controls (see text). The test of blank water control was not significant ($P>0.05$). Treatment groups with different letters are significantly different from each other ($P<0.01$). ***$P<0.001$ (head-to-head test of the holding water of ovulated carp versus the holding water of ovulated goldfish using male carp)

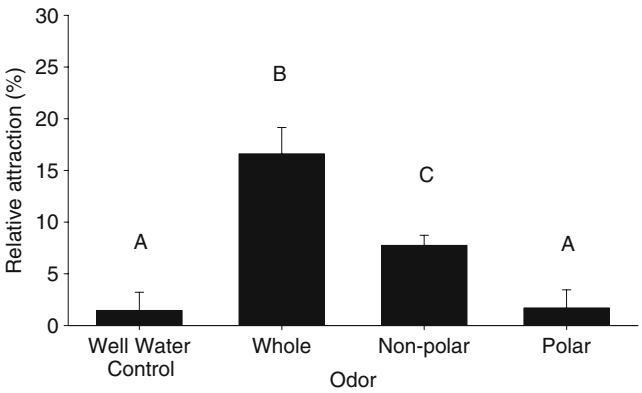


Fig. 3 Responses (mean relative attraction ± standard error) of primed male carp, *Cyprinus carpio*, to blank control, the holding water of ovulated carp ('Whole'), the reverse phase C18 column chromatography eluate ('Non-polar'), and C18 filtrate ('Polar') fractions of ovulated carp water. Samples were tested against appropriate controls (see text). Treatment groups with different letters are significantly different ($P<0.01$)

in each. Quantification of $PGF_{2\alpha}$ / dh15K-$PGF_{2\alpha}$, and 15K-$PGF_{2\alpha}$ using their parent ions (*m/z* 353 and 351, respectively) produced linear calibration curves ($R^2=0.94$; $R^2=0.92$). The latter was used to calculate 15K-$PGF_{2\alpha}$ release rates, which ranged from less than 0.1 ng/g body weight/h for non-ovulated carp to nearly 9.0 ng/g/h for ovulated goldfish (Table 1). Immunoassay further suggested that these species released similar quantities of $PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$, although female goldfish released greater quantities of dh15K-$PGF_{2\alpha}$ (Table 1). When summed, the quantities of $PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$ measured by ELISA were not different from the total values measured by ESI-MS/MS for both products ($P>0.05$), confirming the ELISA results. Relatively high variation in the specific values of individual PGFs released by individual carp and goldfish was evident (Table 1).

Behavioral tests of the carp-specific mixture of $PGF_{2\alpha}$:15K-$PGF_{2\alpha}$: dh15K-$PGF_{2\alpha}$ found it to be attractive when tested against well water control ($P<0.001$) and equivalent in potency to the eluate ($P>0.05$), suggesting it could explain all activity in the non-polar fraction (Fig. 5a). However, when the PGF mixture was tested against whole ovulated carp water, it was less potent than the latter ($P<0.001$), and when added to the filtrate (polar fraction), the potency of the PGF / filtrate mixture was equivalent to that of ovulated carp water (Fig. 5b). Experiments conducted to address our fourth question about PGF ratios showed that of the three PGFs, only $PGF_{2\alpha}$ was attractive ($P<0.05$) at a concentration 2×10^{-10} M ($P<0.001$; Fig. 6a). It was also the only PGF that was active at 6×10^{-10} M ($P<0.001$; Fig. 6b).

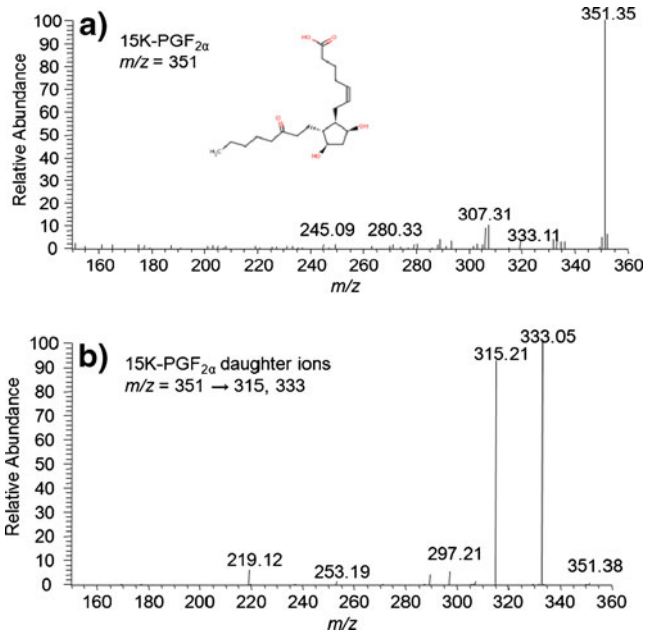


Fig. 4 Mass spectrum of 15 K-$PGF_{2\alpha}$: **a** full scan mass spectrum, and **b** daughter ion spectrum of *m/z* 351. Collision energy was 35 eV

Table 1 Mean release rates[a] of three F prostaglandins by common carp, *Cyprinus carpio*, and goldfish, *Carassius auratus*

Fish	$PGF_{2\alpha}$	15K-$PGF_{2\alpha}$	dh15K-$PGF_{2\alpha}$	Ratio
Non-ovulated carp	0.05±0.02	0.05±0.03	0.04±0.03	1.0: 1.0: 1.0
Ovulated carp	3.43±0.82	5.67±1.02	2.27±0.39	1.0: 1.7: 0.7
Ovulated goldfish	7.57±1.50	8.70±1.05	16.76±1.67	1.0: 1.1: 2.2

[a] Table entries are in ng/g fish weight/h ± standard error

Subsequent head-to-head tests between the carp PGF mixture and the goldfish PGF mixture, and later against $PGF_{2\alpha}$ alone, found them all to be equipotent (relative attraction=1.7± 3.6% and −0.9±1.1%, respectively; $P>0.05$).

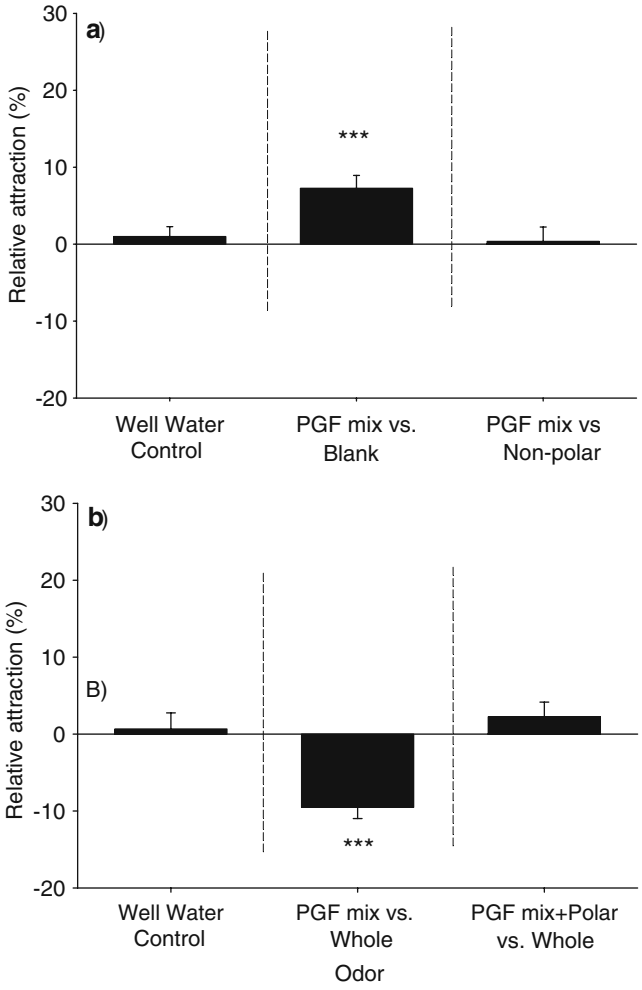


Fig. 5 Responses (mean relative attraction ± standard error) of primed male carp, *Cyprinus carpio*, to: **a** Blank well water control vs. blank well water control; the carp PGF mixture vs. blank well water; and the carp PGF mixture vs. the non-polar eluate of ovulated carp holding water; **b** Blank well water control vs. blank well water control; the carp PGF mixture vs. whole ovulated odor; and the carp PGF mixture vs. the non-polar eluate of ovulated carp holding water plus filtrate vs. ovulated carp holding water. PGF concentrations were matched. ***$P<0.001$

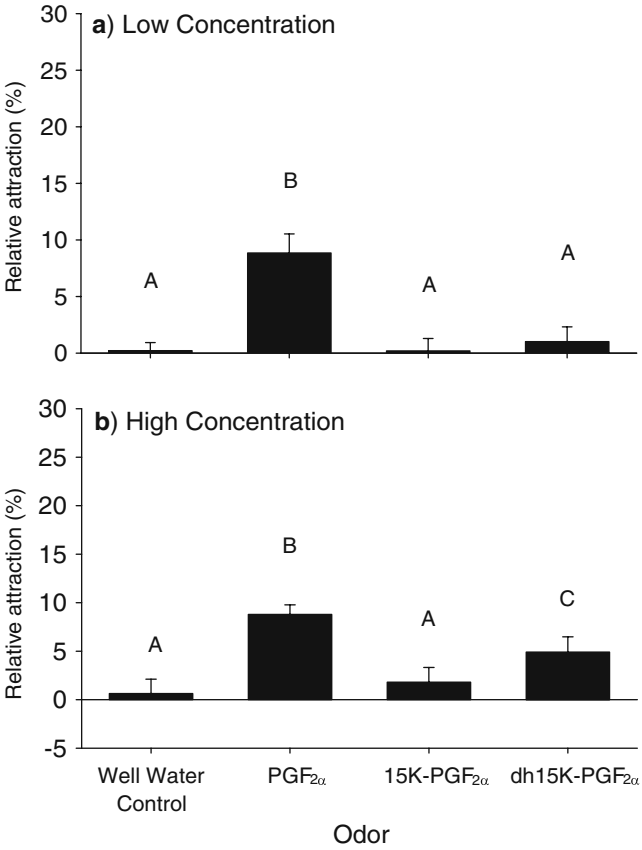


Fig. 6 Responses (mean relative attraction ± standard error) of primed male carp, *Cyprinus carpio*, to the three individual PGFs against well water containing methanol carrier only at: **a** 2×10^{-10} M (the approximate concentrations of each found in ovulated water); and **b** 6×10^{-10} M. Control values did not differ from zero ($P>0.05$). Treatment groups with different letters are significantly different from each other ($P<0.001$)

Discussion

This study is the first to describe the identity and role of PGFs in a pheromone released by an ovulated fish in a definitive manner. It also is the first to describe pheromone release by the common carp, an important model for pheromone function and a damaging invasive species with economic importance. Although we found that $PGF_{2\alpha}$ plays a critical role in the ovulated carp pheromone, we also found that the PGF ratios are seemingly unimportant to the attractive properties of this pheromone and that the activity of $PGF_{2\alpha}$ is synergized by as yet unidentified polar compounds. The latter compounds also may contribute to the species-specific character of this cue, but this has yet to be demonstrated. Because a mixture of different types of compounds is required for the ovulated carp sex pheromone to be fully active, we consider it to be a 'complex.' Further, we hypothesize that the carp has evolved to recognize mature conspecifics by using mixtures of bodily metabolites and relatively common hormonal products such as

PGFs, and that this ability may be shared by other fishes. If so, this could explain the apparent specificity of many (although not necessarily all) hormonal sex pheromones. Additional support for this possibility is found in the observation that male goldfish do not respond to PGFs if they are added to holding water of juvenile blue gourami (*Trichogaster trichopertus*) (Sorensen et al., 2000) and that juvenile goldfish, like mature male carp, are attracted to both polar and non-polar fractions of body washings released by juvenile conspecifics (Levesque et al., 2011). Direct tests of the importance of fish body odors on the perception of hormonal cues and their specificity are now needed to prove this hypothesis.

This study establishes the common carp as an important model for understanding PGF pheromone function while contributing to our understanding of the goldfish. Irvine and Sorensen (1993) showed that the carp and goldfish olfactory systems are acutely and specifically sensitive to $PGF_{2\alpha}$ and two of its metabolites. The present study found that these products are also released in significant quantities by both species, although in slightly different ratios, and that they drive behavioral responses in males of both species. Interestingly, only $PGF_{2\alpha}$ appears to function as an attractant for male carp, even though males also detect 15K-$PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$. There was no indication that unknown PGFs (or other non-polar compounds) contribute to the mixture. Although our study did not directly test whether ovulated carp odor and $PGF_{2\alpha}$ might also stimulate swimming activity and endocrine activity as noted in goldfish (Stacey and Sorensen, 2009), re-review of our data suggested that $PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$ also stimulated swimming activity so we assayed a range of behavioral activities. Although attraction of male goldfish to PGFs has not yet been assayed, they have been shown to become strongly aroused by all three PGFs (Sorensen et al., 1986, 1988, 1989). Thus, PGF function may differ slightly between these species.

Somewhat surprisingly, this study appears to show that the ratios of PGFs released and detected by carp have little bearing on pheromone response in carp. This may make some sense because our studies and those of Ogata et al. (1994) both show PGF release rate and composition to vary greatly and to involve multiple release pathways (Sorensen et al., 1995), which would blur the ratio profiles that fish normally encounter. Additionally, although poorly understood PGF production rates and profiles may change during the course of spawning because of their apparent reliance on the presence of ovulated eggs in the reproductive tract that would change during the course of spawning (Stacey and Sorensen, 2009). The possibility that PGF ratio may influence responses other than attraction (e.g., endocrine responses) is nevertheless untested and warrants attention, both because electrophysiological recording suggests that the goldfish olfactory system has some (albeit limited) ability to discern between them (Sorensen et al., 1988) and because goldfish appear to become more aroused by mixtures of PGFs (Sorensen et al., 1989). The seemingly low importance of PGF release profile contrasts with the situation with the steroidal pre-ovulatory pheromone in goldfish where ratios strongly modulate signal strength; however, the steroids that comprise this cue are released in a highly prescribed temporal sequence that spans many hours, and olfactory sensitivity to them is extraordinarily specific (Scott and Sorensen, 1994; Stacey and Sorensen, 2009). To date, steroid ratio also has not been linked to species-specificity. Many insects are extremely sensitive to small changes in the ratios of pheromonal products but these are produced and released in species-specific ratios from highly specialized glands that apparently evolved and are used for communication alone (Sorensen et al., 1998; Cardé and Haynes, 2004; Derby and Sorensen, 2008). There may be functional relevance to a water-borne sex signal in fish that relies on chemically–unrelated metabolites to convey signal specificity, especially if the main hormonal component ($PGF_{2\alpha}$) is relatively common and there is considerable variation in the means by which it is produced, metabolized, and released.

The failure of male carp to distinguish between PGFs and the non-polar fraction of ovulated carp holding water, together with their preference for holding water (containing both non-polar and polar constituents), suggests that we have isolated all relevant PGs and that their activity is in fact synergized by non-hormonal polar products. Previous studies of the metabolism of radiolabeled $PGF_{2\alpha}$ in goldfish do not support the possibility that the polar products are metabolites of $PGF_{2\alpha}$ (Sorensen et al., 1995, unpublished). We hypothesize that the polar products may be amino acids, which are known to both be released by fish and detected with high sensitivity (Hara, 1994). It is interesting that these unknown polar products were not attractive to sexually active males on their own, suggesting that they provide contextual information whose use may vary with the maturation state of the receiver. These complexes may be used by all life history stages of fish, and their hormonal composition may change with maturational state. Interestingly, recent studies of half a dozen species of fish have now shown that juveniles can discern species identity of other juveniles (Sisler and Sorensen, 2008) and that common polar metabolites make a contribution to this odor complex, which also cannot contain sex hormone products (Levesque et al., 2011). It is conceivable that the same sets of polar compound(s) synergize the activity of the ovulated sex pheromone and convey species information in both juveniles and adults. Thus, species-identity may commonly be conveyed by

complex mixtures of hormonal and non-hormonal (polar) products in fish whose olfactory systems also appear capable of discerning complex olfactory information (Sorensen et al., 1998). Clearly, additional study of the pheromones used by other, more distantly related species is needed to resolve this issue.

The PGF release data collected by this study appear to be the first definitive data on prostaglandin release by a fish. While other studies have employed immunoassay or HPLC, which can be inaccurate, this study used a combination of ESI-MS/MS and immunoassay to both accurately identify the released compounds and quantify their release rates. The masses we identified are both distinctive for PGFs and the values generated for $PGF_{2\alpha}$ and dh15K-$PGF_{2\alpha}$ did not differ from those measured by ELISA, giving us a high level of confidence in our findings. Values previously measured in ovulated goldfish by using radioimmunoassay (Sorensen et al., 1988, 1995) are in fundamental agreement with those generated here, although a bit lower. However, previous studies have used different sized fishes with different numbers of eggs, and we still do not know the precise source of PGFs in fish. Nevertheless, the $PGF_{2\alpha}$ release rates for female carp were adequate to explain $PGF_{2\alpha}$'s behavioral activity in the mazes, given that the carp's olfactory detection threshold for this compound falls between 10^{-10} M and 10^{-11} M (Irvine and Sorensen, 1993; Lim, unpublished data). Notably, release rates of all three PGFs were about 100 times higher by ovulated than non-ovulated carp, making PGFs an excellent and reliable indicator of sexual activity for males. It seems likely that $PGF_{2\alpha}$ is mostly released in the urine of carp as in the goldfish (Appelt and Sorensen, 2007) and that this may enhance their effects in the wild.

In summary, this study establishes that the ovulated common carp, like many fish, release a PGF-based sex pheromone that is both potent and species-specific. Interestingly, although the female carp releases three PGFs, only $PGF_{2\alpha}$ is behaviorally active, and its activity is synergized by unidentified polar metabolites. If other fish also use pheromonal complexes it would explain how combinations of relatively common hormonal products can serve as species-specific pheromones. Research is now needed to identify these complex signals, elucidate their complete function, determine if they are used by other fish species, and to develop them for use as attractants to attract and remove the invasive carp, for which few control measures presently exist.

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Both Gas Chromatography and an Electronic Nose Reflect Chemical Polymorphism of Juniper Shrubs Browsed or Avoided by Sheep

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Abstract Chemical polymorphism may contribute to variation in browsing damage by mammalian herbivores. Earlier, we demonstrated that essential oil concentration in juniper, *Juniperus communis*, was negatively associated with herbivore browsing. The aim of the present study was to characterize the volatile chemical composition of browsed and non-browsed *J. communis*. By using either gas chromatography with flame ionization detection (GC-FID) or an electronic nose device, we could separate sheep-browsed or non-browsed juniper shrubs by their essential oil pattern and complex odor matrix. The main components of the essential oil from *J. communis* were monoterpenes. We distinguished three chemotypes, dominated either by α-pinene, sabinene, or δ-3-carene. Shrubs belonging to the α-pinene- or sabinene-dominated groups were browsed, whereas all individuals with the δ-3-carene chemotype were unused by the local herbivores. The electronic nose also separated the browsed and non-browsed shrubs indicating that their odor matrix could guide sheep browsing. Responses of sheep could integrate the post-ingestive effects of plant secondary metabolites with sensory experience that stems from odor–phytotoxin interactions. Chemotype diversity could increase the survival rate in the present population of *J. communis* as certain shrubs could benefit from relatively better chemical protection against the herbivores.

Key Words Chemical polymorphism · Chemotype · δ-3-carene · Electronic nose · Essential oil · *Juniperus communis* · Gas chromatography-flame ionization detection (GC-FID) · Odor · *Ovis aries* · α-pinene · Sheep

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Introduction

Almost all plants produce secondary metabolites for their chemical defense against herbivores (Freeland and Janzen, 1974; Miller et al., 2005). The quantity and composition of plant secondary metabolites (PSMs) are key factors in plant chemical defense (Villalba and Provenza, 2005), as well as herbivore sensitivity and their coping capacity against toxins (Freeland and Janzen, 1974; Miller et al., 2005; Iason and Villalba, 2006). Plants that contain higher toxin concentrations benefit from relatively high biochemical protection against herbivores (Bryant et al., 1985) or belong to a relatively new and specific PSM polymorphism (Linhart and Thompson, 1999; Provenza et al., 2003). Chemical polymorphism can be manifested in chemotypes, which represent individuals with similar chemical metabolite composition and are usually designated by their main component (Schmidt et al., 2004).

In general, essential oils have strong antibacterial and antifungal effects (e.g., Angioni et al., 2003; Filipowicz et al., 2003). Such oils are repellents for insects such as moths (Rocchini et al., 2000) and beetles (Wibe et al., 1998). Feeding on big sagebrush, *Artemisia tridentata*, which is

high in terpenes, inhibits the cellulolytic activity of rumen microbes (Nagy and Tengerdy, 1968), and accordingly in high volumes it is toxic for ruminants (Schwartz et al., 1980a). Reduced food intake has been associated with plant items containing relatively high essential oil yield in the interaction of mule deer, *Odocoileus hemionus*, with different juniper species (*Juniperus deppeana, J. osteosperma, J. scopulorum*) (Schwartz et al., 1980b) or Spanish goats, *Capra bircus* with two juniper species (*J. ashei, J. pinchotti*) (Riddle et al., 1996).

The ability to discriminate among foods is critical for herbivore survival in an environment abundant in poisonous plants (Provenza et al., 1998). Herbivores must avoid or decrease PSM toxicity (e.g., Provenza, 1995; Dearing et al., 2000; Duncan et al., 2006; Rogosic et al., 2007) and may use the senses of smell and sight before feeding to prevent harmful consequences (Provenza et al., 1992; Provenza, 1996). Domestic sheep, *Ovis aries*, were able to avoid food items treated with common monoterpenes and discriminate them by their odor (Narjisse et al., 1996), and the same was observed in female red deer, *Cervus elaphus* (Elliott and Loudon, 1987). Augner and his colleagues (1998) observed that lambs preferred a weaker flavored food, because the more flavored food was associated with a higher toxin level.

Essential oils, such as the commonly produced PSM group of aromatic plants, are complex natural mixtures (Bakkali et al., 2008). Each component in an essential oil mixture could have a different kind of specific biochemical (toxicological) impact on herbivores (Nagy and Tengerdy, 1968) dependent on its molecule structure and/or its special functional group. Moreover, these components may produce either synergistic or antagonistic interactions with each other (Aparicio et al., 1996; Vokou et al., 2003) influencing the toxicity of the whole mixture. Furthermore, essential oil content shows high diversity with habitat and season (Riddle et al., 1996; Vourc'h et al., 2001; Gomez et al., 2003; Markó et al., 2008) and is also variable among individuals of a particular species (Miller et al., 2005).

Odor or flavor patterns can be measured by electronic sensory systems. Such devices, also called electronic noses, are rapid, objective, and reliable analytical tools for the detection and characterization of volatile and/or odorous compounds (El Barbri et al., 2008). An electronic nose is a detector that contains an array of different sensors and software processing sensor data without reference to the exact chemical composition. Until now, electronic noses have been used to detect and identify volatile compounds (Maricou et al., 1998), and to classify the complex aromatic features of herbs (Novák et al., 2001; Baby et al., 2005; Seregély and Novák, 2005). Their use is widespread in the food and beverage industry (Pearce, 1997b), as well as in quality monitoring (Hobbs et al., 1995; Hogben et al., 2004). The perception of different chemicals also may induce the same taste (Legin et al., 2003) or flavor (Cleland et al., 2002). As browsing animals are not likely to respond to individual chemical components but rather to differences in odor or taste patterns, one would expect a functional similarity in the odor mixture discrimination by an electronic nose and the herbivore (Pearce, 1997a).

Recently, Markó et al. (2008) found that both the concentration of PSMs and specific compounds of the essential oil may play a role in selective browsing damage. The main goal of this study was the characterization of the volatile chemical composition of the common juniper, *Juniperus communis* L., either browsed or non-browsed by sheep. First, we tried to separate the essential oil of differently browsed shrub groups by gas chromatography (GC) to see if browsing was affected by the essential oil composition. Second, we classified the differently browsed shrub groups with an electronic nose reflecting the odor matrix. Third, we compared the odor matrix with the pattern of essential oil composition. Finally, we investigated which terpenoids best reflected the odor matrix.

Methods and Materials

Sampling and Extraction of Essential Oils The samples originated from the juniper forest of the Kiskunság National Park central Hungary, near the area of Orgovány (46°47′30″N; 19°27′34″E). Heavily browsed (N=9) and non-browsed (N=9) shrubs of *J. communis* were chosen, and all plant material was collected at late winter. The sampling procedure was detailed in Markó et al. (2008). The size (height 2–2.5 m) and sex (female) of the sampled shrubs were similar, reducing the possible impact of these factors. The terminal 10–15 cm of foliage was clipped from several branches from ground level to a height of 2 m. Samples were 100–120 g of foliage after removal of berries. Plant material was gathered in plastic bags that were placed immediately in a cooler (5°C), until they were oven dried at 37±3°C (Memmert U-type oven). For the extraction of oil yield, the method of water-distillation with a Clevenger-apparatus described by Pharmacopoea Hungarica (Végh, 1986) was used. The content of essential oil was calculated as percentage of the dry mass.

Gas Chromatography The main chemical compounds of essential oils were analyzed by using a Shimadzu 14B GC equipped with a FID detector and SE-30 capillary column (30 m×0.25 mm, and 0.25 mm film thickness). The carrier gas was nitrogen (1 ml/min), and the split ratio was 75/1. The temperature of the oven was programmed as follows: 110°C for 3 min, up to 220°C at 8°C/min (5 min), 21.75 min analysis time, injector temperature was 220°C

and detector temperature was 250°C. Data were analyzed by using the Shimadzu Class VP Chromatography Data System (Shimadzu, Kyoto, Japan) software. Relative percentage of the oil constituent was calculated from the GC (FID) peak areas as percent of the total area by using the area normalization method (Novák et al., 2001, 2003). The identification of compounds was performed by peak enrichment with GC-standards from Carl Roth (Karlsruhe, Germany).

Electronic Nose Analysis Electronic nose determinations were performed with an NST 3320 instrument (Applied Sensor, Linköping, Sweden), with a built-in headspace autosampler for 12 samples, a detector unit containing 22 different sensors, and software for collecting and processing data from the sensors. The NST 3320 is equipped with 10 MOSFET (metal oxide semiconductor field effect transistor) sensors, 12 MOS (metal oxide semiconductor) sensors, and a humidity sensor for measuring relative humidity. Ambient air filtered through a drying column with silica gel and a combined moisture/hydrocarbon filter was used as a clean reference gas for the sensors. The gas flow rate of the dynamic sampling was set to 60 ml/min. The filtered gas flow to the sensors, and into the headspace of the samples at the same time, finally left the instrument into the ambient air.

The stand-by temperature, at which the samples were kept until their incubation phase, was set to 20°C (5 min). The analysis was started with the incubation at 40°C (5 min). The baseline phase of the measurement cycle timing was set to 10 sec, the sample phase to 30 sec, the recovery phase of the sensors to 260 sec, and the flush time of the gas lines to 60 sec. The total cycle time per sample was 5 min. Signal parameters were collected as follows: "baseline" for 5 sec, "on derivative" (derivative of the sensor signal during adsorption) for 4 sec and "on integral" (integral of the sensor signal during adsorption) for 10 sec, "response" for 5 sec, "off derivative" (derivative of the sensor signal during desorption) for 4 sec and "off integral" (integral of the sensor signal during desorption) for 10 sec. Sample amount and temperature conditions for the electronic nose analysis were optimized before the actual quality evaluations in the storage test.

We used 0.7 g of fresh juniper twigs from the same shrubs as described above for the GC-FID analysis. After removing the berries, twigs were crushed and put into the labeled vials, which were sealed with a Teflon-coated butyl septum and a screw cap. Electronic nose measurement was conducted at the Department of Refrigeration and Livestock Products Technology, Corvinus University of Budapest. The parallel analysis of samples collected from the same juniper shrubs allowed us to compare the results of the GC and the electronic nose analysis.

Statistical Analyses

Multivariate Statistical Analyses For the evaluation of the electronic nose sensor signal response of the sensor array, principal component analysis (PCA) was performed. The 22 sensor values were reduced to three principal components. Also, PCA was performed using the data set composed of all the essential oil samples transformed into centered and reduced variables (correlation method, varimax rotation). Selected variables consisted of absolute amounts of the identified compounds in the essential oil samples. The PCA is sensitive to rare or missing values, so the components less than 1% of the total samples were excluded from the analysis. The volume of each component was calculated from its relative ratio to the essential oil yield of each shrub. The use of absolute concentrations rather than relative amounts demonstrates that such calculations are not a statistical artifact of compositional data. In this way, we corrected for the total essential oil yield, which it is more realistic in sheep-juniper interactions.

Factor analysis was used to determine the main variables for each PC of the essential components. The scores from the PCA were inserted to the factor analysis. The first three factor loadings were included based on the eigenvalues (cumulative variance 85.07% in GC-FID).

General Linear Mixed Model After PCA, we used general linear mixed models (GLMM) to investigate the relationship between browsing status and the first three PCs of the electronic nose. Browsing status was a dependent variable and showed binomial distribution, while the PCs were used as covariates. We used GLMM to investigate the relationship between browsing status of the juniper shrubs and the first three PCs from GC-FID analysis. Browsing status was a dependent variable and showed a binomial distribution, while the PCs were included as covariates in the model.

In all GLMM models, we employed a stepwise analysis with a backward deletion procedure, removing insignificant effects from the model one by one in decreasing order of statistical significance and re-entering the removed variables to the final model to obtain relevant statistics. Statistica package (version 8.0, StatSoft, Inc. 2004, Tulsa, OK, USA) was used for all statistical analyses.

Results

Classification by Essential Oil Patterns We extracted essential oils from *J. communis* and identified their components (Table 1). The PCA distinguished the individual plants based on their browsed level with high power (85.07%) in the first three PCs, which are presented on

Table 1 Composition of the essential oils of the three chemotypes of *Juniperus communis* from Hungary

Components	Chemotype								
	δ-3-carene (*N*=4)			Sabinene (*N*=13)			α-pinene (*N*=1)		
	Mean (μl)	± SD	CI	Mean (μl)	± SD	CI	Mean (μl)	± SD	CI
α-Pinene	41.36	±5.61	3.18–20.93	46.33	±37.78	27.1–62.38	81.63		
Sabinene	0			121.20	±92.03	66–151.93	3.04		
δ-3-Carene	239.9	±117.64	66.65–438.66	3.54	±2.45	1.76–4.05	0		
β-Myrcene	24.16	±9.49	5.38–35.39	13.70	±8.47	6.08–13.99	3.77		
γ-Terpinene	20.28	±4.31	2.44–16.09	14.68	±6.42	4.61–10.61	21.05		
Borneol	21.65	±8.51	4.82–31.74	14.68	±8.57	6.15–14.15	0		
cis-Nerolidol	0			1.80	±2.62	1.89–4.34	0		
trans-Neroidol	9.04	±6.13	3.48–22.89	7.90	±6.31	4.53–10.42	1.95		
γ-Muurolene	0			3.74	±5.11	3.67–8.44	0		
Total volume of the essential oil	431.06	±170.06	96.34–634.09	283.36	±162.92	116.83–268.95	121.65		

Columns contain the mean values, standard deviations (SD) and confidence intervals (CI) of each chemotypes, which were calculated from the given sample size (*N*). The rows contain the main essential oil components

scatter plots with each other (Fig. 1b). The correlation plot of PC 2 and PC 3, and plot of PC 2 and PC 3 showed well classified groups. The scores of browsed shrubs were more aggregated than the values for the non-browsed junipers, similarly to the electronic nose classification. Furthermore, if we compare the scatter plots of the two patterns (Fig. 1a and b), we find the essential oil PCs less aggregated than the dots from the electronic nose analysis.

The GLMM analysis (Table 2) supports the PCA classification of the essential oil pattern. The PC 2, PC 3 and their interaction were significantly important in discrimination of the juniper individuals, while PC 1 was not significant in the model.

Factor Analyses of Gas Chromatography After PCA, reduction factor analyses was used to see which essential oil components belonged to each PC (Table 4). The first factor (PC 1) included almost all monoterpene components, e.g., β-myrcene, γ-terpinene, and borneol, whereas the dominant monoterpenes, such as α-pinene, sabinene, and δ-3-carene, composed the third factor (PC 3). The second factor (PC 2) grouped all the sesquiterpenes. The essential oil components showed that their retention times were close to each other. The retention time is reflected by the structure and weight of each molecule.

Sensory Classification by the Odor Matrix We measured the odor pattern of browsed and non-browsed *J. communis* by using an electronic nose apparatus; the sensor values were analyzed with PCA; the first three principal component (PC) scores were displayed in a scatter plot (Fig. 1a). Based on their browsed status, we could classify the odor patterns with high power (77.1%). The scatter plots between PC 1 and PC 3, and between PC 1 and PC 2 express the plant use best. These plots show that the browsed groups were well demarcated from the non-browsed groups, although there were three browsed individuals (numbers 2, 5, and 6) that seemed more similar to the non-browsed groups than to the browsed ones. The plots of PC 2 and PC 3 showed less determinant differences among the groups. Furthermore, the correlation plots showed that scores of browsed juniper individuals were more aggregated than the non-browsed junipers.

The GLMM analysis (Table 3) supported the PCA classification. Every PC was significantly important in the discrimination of the juniper individuals, and none of the interactions was significant in the model.

Discussion

We investigated the essential oil pattern of previously sheep-browsed and non-browsed *J. communis* and found that both the electronic-nose (e-nose) and GC separated the two groups. By using the e-nose, we also found that the juniper shrubs contained significantly different odor patterns depending on their browsed status. For studying the relationship between browsed status and essential oil patterns, we reduced the number of components by PCA. The factor analysis suggested that browsed and non-browsed shrubs differed in their mono- and sesquiterpene

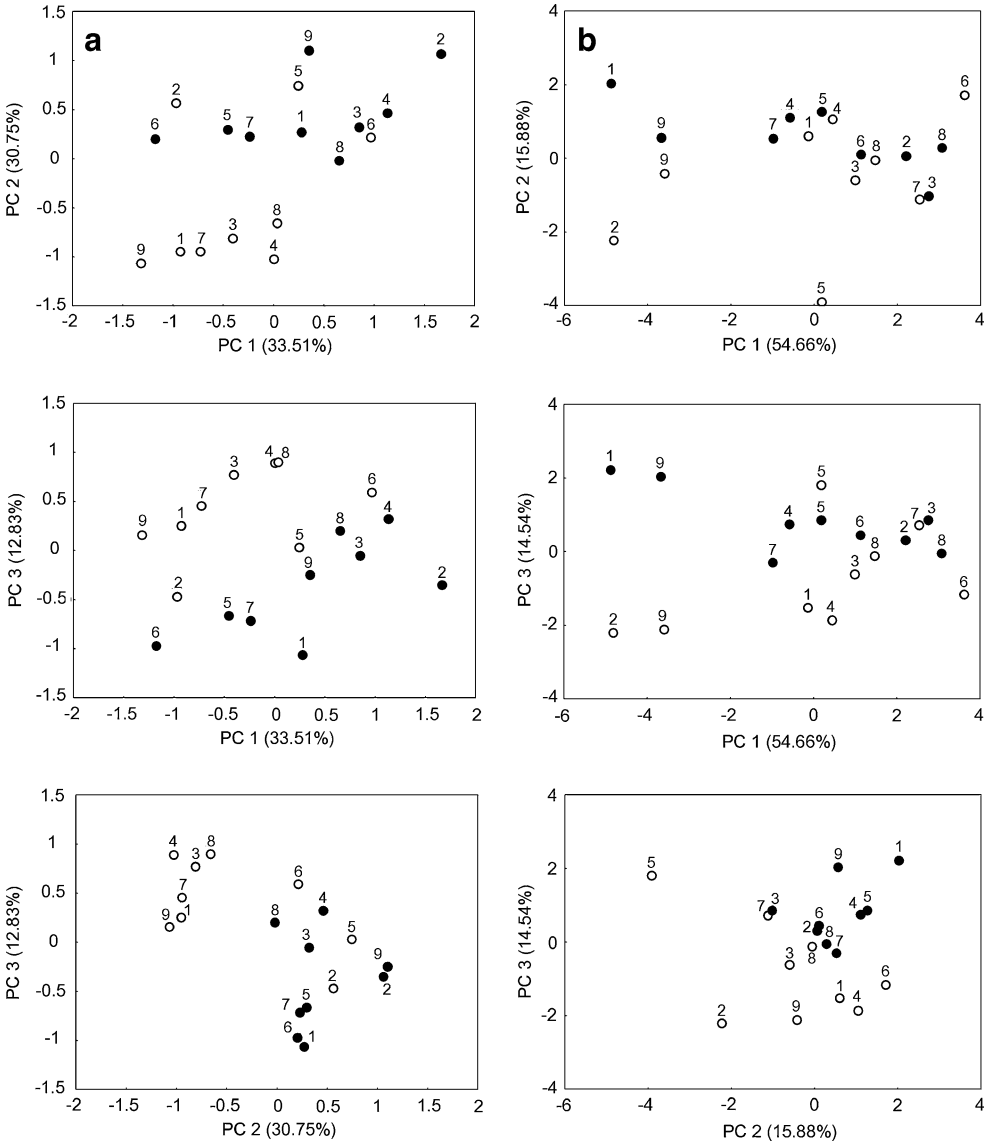


Fig. 1 Scatter plots of principal components (PC) showing the electronic-nose (**a**) and GC (**b**) discrimination of the juniper shrubs, *Juniperus communis*. The lines enclose the browsed (*empty circles*) and non-browsed (*full circles*) individuals, respectively; labels describe samples. The cumulative power of electronic-nose was 77.1% for the first three factors, whereas it was 85.1% for the GC. The browsed groups were well demarcated from the non-browsed groups, but some browsed shrubs (ID: 2, 5, 6) showed non-browsed odor characteristics in both classification methods

ratios (see Table 4). We distinguished three different chemotypes, dominated by α-pinene, sabinene, and δ-3-carene, respectively, in our juniper population. All shrubs belonging to the α-pinene were browsed, whereas all δ-3-carene chemotype individuals were unused by the local herbivores. Shrubs containing elevated levels of sabinene were mixed in their browsed status. However, the δ-3-carene chemotype has been reported for Scots pine, *Pinus sylvestris* L. (Thoss et al., 2007), but to our knowledge this

Table 2 *F*-statistics of GLMM analyses of gas-chromatography

	F value	*d.f.*	*P*
PC 1	0.02	1, 14	0.873
PC 2	4.64	1, 15	0.047*
PC 3	10.53	1, 15	0.005**
PC 2:PC 3	1.24	1, 14	0.232

F-statistics of GLMM analyses are shown. Principal components (PC) are from the PCA of gas-chromatography. Stars indicate the significance level among factors (*—$P<0.05$; **—$P<0.01$). The model had the statistics $F=7.591$, *d.f.*=2,15, $R^2=0.436$, $P=0.0052$

Table 3 *F*-statistics of GLMM analyses of electronic nose

	F value	*d.f.*	*P*
PC 1	8.29	1,14	0.012*
PC 2	10.76	1,14	0.005**
PC 3	15.37	1,14	0.001**
PC 1:PC 2	0.24	1,13	0.628
PC 1:PC 3	1.00	1,13	0.334
PC 2:PC 3	0.04	1,13	0.828
PC 1:PC 2:PC 3	0.49	1,13	0.492

F-statistics of GLMM analyses are shown. Principal components (PC) are from the PCA of the electronic nose. Stars indicate significance the level among factors (*—$P<0.05$; **—$P<0.01$). The model had the statistics $F=11.48$, *d.f.*=3,14, $R^2=0.649$, $P<0.001$

Table 4 Composition of the essential oils of the three factors in browsed *Juniperus communis* from Hungary

Ret. time	Component	Terpene group	Factor 1	Factor 2	Factor 3
8.75	Unknown	Monoterpene?	−0.964*	0.080	−0.044
9.02	α-Pinene	Monoterpene	−0.284	0.267	−0.660*
10.08	Sabinene	Monoterpene	−0.433	−0.473	−0.705*
10.15	δ-3-Carene	Monoterpene	−0.569	0.474	0.620*
10.44	β-Myrcene	Monoterpene	−0.985*	0.076	0.021
11.14	Unknown	Monoterpene?	−0.988*	−0.059	0.002
11.23	Unknown	Monoterpene?	−0.662*	−0.311	−0.445
11.51	γ-Terpinene	Monoterpene	−0.659*	0.487	−0.266
12.22	Unknown	Monoterpene?	−0.888*	0.083	0.280
12.98	Unknown	Monoterpene?	−0.966*	−0.008	0.118
18.03	Borneol	Oxigenated monoterpene	−0.883*	−0.259	0.191
23.61	*trans*-Nerolidol	Oxigenated sesquiterpene	−0.471	−0.701*	0.310
24.76	γ-Muurolene	Sesquiterpene	0.180	−0.792*	0.269
	Expl. variance		7.105	2.064	1.889
	Prp. total		0.546	0.158	0.145

The components are quoted by their retention order of GC analysis. The most important factors are indicated by stars (*) in each essential oil component

is the first study to identify uncommonly high amount of this monoterpene in *J. communis*.

The observed juniper population was rich in monoterpenes (Markó et al., 2008) similarly to other stands of this species (e.g., Adams and Pandey, 2003; Butkiene et al., 2004). The factor analysis of the essential oil pattern determined by GC resulted in three factors: F1 contained monoterpenes with medium retention times; F2 contained sesquiterpenes; whereas F3 was related to dominant monoterpenes. The relative importance of the dominant monoterpenes and the sesquiterpenes differed significantly between the two browsed statuses (Table 1). The first group included almost all monoterpene components, the second group incorporated all identified sesquiterpenes, while the dominant monoterpenes, such as α-pinene, sabinene, and δ-3-carene, composed the third group. Essential oil mixtures can show high terpenoid diversity containing 20–60 different components at quite different concentrations (Bakkali et al., 2008) and quite different toxicities (Oh et al., 1967), so grouping the components to find common patterns seems to be necessary to understand their effect in reducing herbivory.

Juniperus communis exhibits chemical polymorphism with geographical variation (Adams et al., 2003). A Swedish population showed high amounts of α-pinene, with moderate amounts of β-pinene, myrcene, limonene, δ-3-carene, and β-phellandrene, and low amounts of sabinene (Adams, 1998). Sabinene-type and α-pinene-type junipers have been described for Poland (Filipowicz et al., 2009) and Lithuania (Butkiene et al., 2007, 2009). The δ-3-carene has been described in many other juniper species (Adams, 1998; Angioni et al., 2003) and also in pines (Rocchini et al., 2000; Hiltunen and Laakso, 1995). PSMs, such as δ-3-carene, could be effective toxins, having a relatively unique molecular structure, because herbivores hardly ever cope with it. Despite these widely existing chemicals among conifers, plants might use them in their defense systems. The oil of *J. communis* with high carene content is effective against bacteria and fungi species (Angioni et al., 2003), and the same effect has been observed in insects (Rocchini et al., 2000; Almquist et al., 2006). δ-3-Carene has shown strong inhibition on the rumen microbial activity in sheep (Oh et al., 1967).

PSM production and composition are well studied in conifers. Among conifer taxons, terpenoid biosynthesis shows high similarity and the genetic regulation also is known (Trapp and Croteau, 2001; Fäldt et al., 2003). Terpene production and the relative amounts are genetically determined and expressed through specific enzymes, monoterpene synthetases (Huber et al., 2004; Martin et al., 2004). Thoss and colleagues (2007) found two different chemotypes, either δ-3-carene or α-pinene dominated, for *P. sylvestris*. They also found δ-3-carene production to be negatively associated with α-pinene concentration. The same biochemical trade-off between δ-3-carene and α-pinene was found in *J. communis* (Orav et al., 1997), similar to junipers of the chemotype in the present study. The δ-3-carene concentration is under strong genetic control (Hiltunen, 1975; Yazdani et al., 1982; Kinloch et al., 1986). In *Pinus taeida* L., variation in content of all major monoterpenes except α-pinene, such as β-pinene, myrcene, limonene, and β-phellandrene, seems to be controlled by two alleles at a single locus (Squillace et al., 1980).

In the present study, browsed and non-browsed juniper shrubs contained different odor patterns. We found that every δ-3-carene chemotype shrub was untouched. This chemotype contains a special essential oil composition pattern, which could be more aversive or toxic to sheep than the other chemotypes, such as α-pinene or sabinene. Chemical quality

of the essential oil can be linked to its unique odor matrix (Novák et al., 2001). We also should note that some browsed junipers (shrub ID: 2, 5, 6, see Fig. 1a) showed non-browsed odor characteristics. Supposing that the discrimination ability of sheep and our electronic nose is similar, there are two alternative hypotheses to explain this category overlap: browsing of junipers could be based on complex odor pattern recognition and selection, or avoidance of one specific component. Neurological bases of mammalian olfaction enabling pattern recognition are well known (Buck and Axel, 1991; Vosshall et al., 2000), and their importance in sheep also has been demonstrated (Lledo et al., 2005). Such similarity in the categorization of junipers by sheep and electronic nose suggests that discrimination in both sheep browsing and in the electronic nose analysis may have common bases.

The sensing ability of sheep is crucial for avoiding unpalatable or poisonous plants and for survival (Provenza et al., 1998). Unpalatable food plants often have strong or unpleasant odors and/or taste (Eisner and Grant, 1981). Sheep are able to discriminate different kind of aromatic food items, containing volatile compounds. In an aversion learning study, Augner et al. (1998) found that they preferred weaker flavors over strong ones, because the animals learned to associate the flavor with toxicity and avoided the conditioned flavor depending on its concentration. Narjisse et al. (1996) reported that sheep exposed to the aroma of a monoterpene mixture during feeding discriminated against feed associated with the terpene mixture. Other ruminants also are able to discriminate odors on the basis of the quality of their food. Red deer rejected a pelleted diet while being exposed to the odor of widespread monoterpenes, including α-pinene (Elliott and Loudon, 1987). Monoterpenes also have been shown to be related to shrub use by goats (Riddle et al., 1996). Estell et al. (1998) found that the negative influences of specific monoterpene (α-pinene and camphor) concentrations on pellet consumption could explain differential herbivory of individual tarbush plants.

Herbivores may have different kinds of effective, non-exclusive mechanisms to avoid poisoning. We propose several alternative explanations for the avoidance of δ-3-carene chemotype individuals. The first may be that sheep food choice is conservative, thus the new chemical variant could be an unfamiliar food option for them. As Van Tien and his colleagues (1999) demonstrated, sheep readily eat foods with a familiar odor, but if an unfamiliar food is presented, the animals show aversion. The second explanation could be that the δ-3-carene dominated individuals are relatively new phenotypic variants among junipers in our stand. Therefore, herbivores have not yet developed defense mechanisms to cope with this special and potent toxin mixture. The chemical polymorphism in the studied juniper population might indicate an ongoing adaptation against local herbivory (Markó et al., 2008), because it might provide relatively higher protection against local herbivores unique in consuming junipers.

Varying enantiomeric composition of certain monoterpenoids could be a third alternative explanation for the relationship of differential browsing and odor characteristics. Several studies have proven the importance of enantiomers on the effect of odors (Friedman and Miller, 1971; Leitereg et al., 1971; Laska et al., 1999) or their influence on other biological activities (Norin, 1996; Wibe et al., 1998; Filipowicz et al., 2003). Enantiomeric composition of monoterpenes has been investigated in *J. communis*, and α-pinene, sabinene, and myrcene were found as the most varied components (Ochocka et al., 1997). In other juniper populations, δ-3-carene occurred almost entirely as the (+)-enantiomer, whereas the enantiomeric composition of other monoterpenes was highly variable (Hiltunen and Laakso, 1995; Filipowicz et al., 2006). The same pattern was found in *P. sylvestris*, where large differences were found in the enantiomeric ratios among monoterpenes, but in δ-3-carene only the (+)-enantiomer was detected (Sjödin et al., 1996).

Nutrients and PSMs do not act in isolation in determining the level of herbivory (Villalba and Provenza, 2005). Partial preferences for certain plant species have been attributed to the inability of herbivores to discriminate among related plant species (Illius et al., 1999), limited perception (Berec, 2000), attempts to meet nutritional needs (Westoby, 1978), or reducing intake of poisonous food (Freeland and Janzen, 1974). The emerging evidence that integrates biochemical interplay between plant species and browsers suggests that varied diets result from the experience with nutrients and toxins (Provenza, 1995; Provenza et al., 2003). Responses of herbivores integrate the post-ingestive effects of PSMs with sensory experience that results in odor–nutrient–phytotoxin interactions.

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Synthesis and Field Tests of Possible Minor Components of the Sex Pheromone of *Prionus californicus*

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Abstract Earlier work has shown that adult male *Prionus californicus* Motschulsky (Coleoptera: Cerambycidae) are attracted to the female-produced compound (3*R*,5*S*)-3,5-dimethyldodecanoic acid, and to a synthetic mixture of the four stereoisomers of 3,5-dimethyldodecanoic acid. Here, we report the results of field trials that tested whether or not three structurally related compounds (methyl 3,5-dimethyldodecanoate, 3,5-dimethyltridecanoic acid, and 3,5-dimethylpentadecanoic acid), present in extracts of virgin females, are attractive, and whether or not they influence attraction to 3,5-dimethyldodecanoic acid. In a trial with single components, only traps baited with the acid or its methyl ester captured more beetles than did control traps; catches to the acid were five times higher than to the methyl ester. Another trial, excluding 3,5-dimethyldodecanoic acid, confirmed the activity of the methyl ester. Finally, addition of the three compounds to 3,5-dimethyldodecanoic acid, in the ratio found in extracts from female beetles, gave a catch similar to that of traps baited with 3,5-dimethyldodecanoic acid alone. Consequently, the function of these minor compounds remains undetermined.

Key Words Prioninae · Pest management · Pheromone · (3*R*,5*S*)-3,5-dimethyldodecanoic acid · Coleoptera · Cerambycidae

Introduction

A number of insects including the herald moth, *Scoliopteryx libatrix* L, and the peach twig borer, *Anarsia lineatella* Zeller, produce novel, branched alkanes, alkenes, and alcohols (Francke et al., 2000; Schlamp et al., 2005). Adult females of the cerambycid beetle *Prionus californicus* Motschulsky produce a volatile sex pheromone, (3*R*,5*S*)-3,5-dimethyldodecanoic acid, to which males are strongly attracted, and which has potential as a management tool (Rodstein et al., 2009, 2011; Maki et al., 2011). Males responded as strongly to a synthetic mixture of the four stereoisomers of 3,5-dimethyldodecanoic acid as they did to the natural enantiomer, indicating that the unnatural stereoisomers were not inhibitory (Rodstein et al., 2011). Antennae of male *P. californicus* responded most strongly to 3,5-dimethydodecanoic acid in coupled gas chromatography-electroantennography (GC-EAD) studies, and also responded to three other compounds, the methyl ester of 3,5-dimethyldodecanoic acid and the homologs 3,5-dimethyltridecanoic acid and 3,5-dimethylpentadecanoic acid, that were present in solid phase microextraction (SPME) wipe samples of ovipositors

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(Rodstein et al., 2009). Here, we report field trials testing these compounds.

Methods and Materials

Field sites were commercial hop yards in Canyon Co., ID, USA, which were infested with *P. californicus*. We used pitfall traps made of polypropylene buckets (19 l, 38 cm tall×30 cm diam.) fitted with aluminum funnels (model 2815B, BioQuip, Rancho Dominguez, CA, USA). Traps were buried between rows of hop plants, with tops of funnels flush with the soil surface, at least 27 m from the margins of hop yards. We conducted three independent bioassays at different times during 3–15 July 2009 (max. air temp: 28–36°C; average wind speed: 2.4–15.5 $km.h^{-1}$; 0.18 cm of precipitation on 15 July).

In Bioassay 1, we compared the attractiveness of the four individual compounds. Compounds were synthesized non-stereoselectively as mixtures of all four possible stereoisomers in approximately equal proportions (≥90% chemical purity), using the methods of Rodstein et al. (2009), and described in the online Supplementary Information. Lures were clear, low-density, polyethylene press-seal bags (Bagette model 14770, 5.1×7.6 cm, 0.05 mm wall thickness, Cousin Corp., Largo, FL, USA), with 10 mg of synthetic compounds, diluted to 100 µl in hexane (HPLC grade, Chromasolv®, Sigma-Aldrich, St. Louis, MO, USA), or 100 µl hexane alone (controls). Lures were attached to the wire bucket handles, which had been modified to remain upright. Treatments were randomly assigned to traps, positioned 18 m apart in each of three commercial hop yards, which were at least 0.4 km apart. The bioassay was repeated for another 3 d in two of the hop yards, re-randomizing treatments daily, to yield nine replicates. Each morning we recorded the number of male *P. californicus* trapped during a night. Differences between treatments in the number of beetles caught were tested with the nonparametric Friedman's test (blocked by day and hop yard; Proc Freq with CMH option; SAS Institute, 2001). We tested differences between pairs of means with the REGWQ means separation test to control maximum experiment-wise error rates (SAS Institute, 2001).

Because 3,5-dimethyldodecanoic acid could affect attraction of males by the other compounds, either positively (by attracting beetles into the vicinity of traps) or negatively (by competing against the other compounds), we compared the activity of 3,5-dimethyldodecanoate, 3,5-dimethyltridecanoic acid, and 3,5-dimethylpentadecanoic acid, in the absence of 3,5-dimethyldodecanoic acid, in Bioassay 2. We used the same methods and hop yards as in Bioassay 1. The bioassay was replicated in two hop yards over 4 d, re-randomizing treatments daily, to yield eight replicates. Data were analyzed as above, excluding one replicate that caught no beetles.

In Bioassay 3, we compared the activity of 3,5-dimethyldodecanoic acid alone vs. a blend of all four compounds that approximated their proportions in extracts of ovipositors (Rodstein et al., 2009). For the latter treatment, lures were loaded with 100 µl of a stock solution of 100 mg of 3,5-dimethyldodecanoic acid, 2.4 mg of methyl 3,5-dimethyldodecanoate, 36 mg of 3,5-dimethyltridecanoic acid, and 133 mg of 3,5-dimethylpentadecanoic acid, diluted to 1 ml in hexane. We used the methods described above, replicating the experiment in four commercial hop yards (including two used in the other bioassays). Treatments were re-randomized daily for 3 days and on a 4th day in one yard, yielding 13 replicates. Differences between treatments in catches of male *P. californicus* were tested with a paired *t*-test, after the normality of data was confirmed using the Shapiro-Wilk test for normality (SAS Institute, 2001). We present means ± 1 SE throughout.

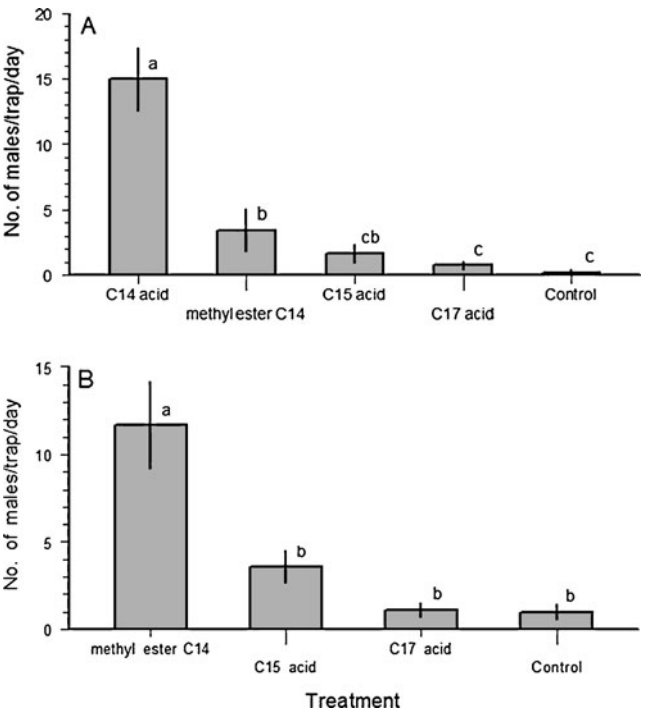


Fig. 1 Mean (± 1 SEM) numbers of male *Prionus californicus* captured by traps baited with various compounds (as mixtures of stereoisomers) that are released by females: **A**) Bioassay 1, **B**) Bioassay 2. Compounds tested were: 3,5-dimethyldodecanoic acid (C14 acid), methyl 3,5-dimethyldodecanoate (methyl ester C14), 3,5-dimethyltridecanoic acid (C15 acid), and 3,5-dimethylpentadecanoic acid (C17 acid). Control traps had lures of hexane only. Bars with the same letters are not different (REGWQ means-separation test) at $P<0.05$

Results and Discussion

No female *Prionus californicus* were captured in any bioassay. In Bioassay 1, only traps baited with 3,5-dimethyldodecanoic acid or its methyl ester captured more male *P. californicus* than did controls. Traps baited with the acid captured more than five times as many beetles as those baited with the methyl ester (Fig. 1A; Friedman's $Q_{4,45}$= 27.9, P<0.001). The activity of the methyl ester, in the absence of 3,5-dimethyldodecanoic acid, was confirmed in Bioassay 2 (Fig. 1B; Friedman's $Q_{3,28}$=115.1, P<0.002). These results suggest that the methyl ester is moderately attractive to male beetles, consistent with a stronger antennal response in GC-EAD analyses to that compound, relative to homologs of the acid (Rodstein et al., 2009).

Despite the activity of the methyl ester when tested alone, the reconstructed blend of components that mimicked the ovipositor extracts was no more attractive to males than 3,5-dimethyldodecanoic acid alone (means: 8.3±1.9 and 9.0±2.1 males/trap/day, respectively; not different, paired t-test P= 0.45). Although it is possible that one or more of the pure stereoisomers of these analogs might affect attraction to 3,5-dimethyldodecanoic acid, this seems unlikely, as any antagonistic effect from a particular stereoisomer would likely be manifested despite the presence of its stereoisomers. Similarly, it is unlikely that any synergistic effect from a particular stereoisomer would exactly cancel out effects by one or more of the other stereoisomers.

That possible minor components have no apparent effect on attraction of beetles to 3,5-dimethyldodecanoic acid, coupled with the fact that the unnatural stereoisomers of (3*R*,5*S*)-3,5-dimethyldodecanoic acid have no effect on its activity (Rodstein et al., 2011), will simplify development of the pheromone for management of *P. californicus*.

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Signal Honesty through Differential Quantity in the Female-Produced Sex Pheromone of the Moth *Heliothis virescens*

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Abstract Over the last 50 years, female-produced sex pheromones of moths have been subjected to intensive study. Most work has focused on their role in mate recognition, and little on any role they may have in mate assessment. This is largely because it has been assumed that female, rather than male, moths are "choosy", and invest larger amounts of carbon in eggs than in pheromone. Recently, we found that pheromone production in the moth *Heliothis virescens* depended on hemolymph trehalose concentration, and that sugar-stressed females produced less pheromone than unstressed ones. In this paper, we demonstrate, for the first time in moths, that a female-produced pheromone signal can allow *H. virescens* males to assess sugar resources (quality) of a female. This signal honesty is based on quantitative, rather than qualitative (component ratio), differences in pheromone, produced and released by sugar-stressed and unstressed females. Increasing marginal cost of pheromone production, as sugar resources are depleted, may ensure signal honesty.

Key Words Chemical signaling · Hemolymph trehalose · Mate assessment · Lepidoptera · Noctuidae

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Introduction

Chemical signals can be used to recognize mates or, more profoundly, to choose a mate based on fitness benefits the signal receiver may obtain from mating (Johansson and Jones, 2007). A key principle of the latter is that the signal should honestly represent the differential quality of the signaler, allowing a receiver to choose a mate to maximize his/her fitness (Hausken and Hirshleifer, 2004). Most work on pheromone mating signals has focused on their role in recognition, although examples (reviewed in Johansson and Jones, 2007) in which they enable mate assessment are known. This work on mate assessment in mating signals typically has focused on female assessment of male quality (e.g., Iyengar et al., 2001; Lassance and Löfstedt, 2009) because, in sexual selection theory, females are generally considered the "choosy" sex, investing more resources in reproduction than do males (Trivers, 1972). However, mate assessment by males is known in animals (Bonduriansky, 2001), and in insects it is known to occur through assessment of female size (e.g., Rhainds et al., 1995; Van Dongen et al., 1998), which typically correlates with fecundity (e.g., Xu and Wang, 2009).

Female-produced sex pheromones of moths are legendary for specificity and potency at minute concentrations (Wyatt, 2003). In most moth species, individual isolated females release sex pheromone that attracts conspecific males. The chemistry of, and perception and behavioral responses of males to, these signals have been studied intensively (Witzgall et al., 2010) within the context of mate recognition (Johansson and Jones, 2007). While a number of studies have shown that male moths can discriminate among females, based on pheromone compo-

nent ratios (e.g., Collins and Cardé, 1989; Allison and Cardé, 2008), none has shown that male discrimination of signals among females has fitness consequences.

Recently, we found that pheromone production in mated and virgin females of the polyandrous moth, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae), depended on feeding status (Foster, 2009; Foster and Johnson, 2010). Adults of this species, like many other Lepidoptera, feed on plant nectar (Ramaswamy et al., 1997; Wäckers et al., 2007). Sugar feeding resulted in an increase in hemolymph trehalose concentration (HTC) that, in turn, caused increases in pheromone and fatty acid precursors. This effect occurred only in sugar-stressed females (i.e., with low HTC; Foster, 2009), caused by prolonged starvation or mating, the latter lowering HTC through increased oocyte maturation (Ramaswamy et al., 1997; O'Brien et al. 2000).

As nectar feeding causes large increases in fecundity of females of *H. virescens* (Ramaswamy et al., 1997), and in Lepidoptera in general (Wäckers et al., 2007), we hypothesized that quantitative pheromone differences between sugar-replete and sugar-stressed females may be perceived by males, and thus might honestly signal female quality. In this manuscript, we demonstrate, for the first time, that the sex pheromone produced by a female moth acts as an honest signal of female quality (sugar resources) and potential fitness benefits to males. This honesty is conveyed quantitatively (amount) rather than qualitatively (component ratios).

Methods and Materials

Insects Insects were from a colony originally supplied by Dr W.L. Roelofs (Cornell University, Geneva, NY, USA). Larvae were reared on a wheat germ-casein diet at 25°C under a 16:8 h L:D photoperiod. After pupation, insects were sexed, and females and males kept in separate incubators under the same conditions as larvae.

Adult females, 4–5 per container, were given either 10% sucrose solution or water, on cotton wool, to feed. The liquid was changed daily. Mated females were obtained by placing 1-d-old virgins with an excess of virgin males (2-3-d-old). Females that mated were placed in a container with their previous food (sugar or water) and used 2 d after mating (when 3-d-old). Sugar-stressed virgins were obtained by allowing females to feed on water for 4 or 5 d (Foster and Johnson, 2010). Younger virgins (1- or 3-d-old), fed on water or sugar, were used as unstressed controls.

Pheromone Analyses The pheromone gland of a female was dissected at one of three times in the scotophase: beginning (0–0.5 h), mid (3.75–4.25 h), and end (7.5–8 h). The gland was extracted in 10 µl *n*-heptane, with 50 ng of (*E*)-11-tetradecenal as internal standard, for at least 3 h at ambient temperature before analysis by gas chromatography–mass spectrometry (GC-MS) on a Hewlett-Packard 5890–5972 system, fitted with a 30 m×0.25 mm i.d. ZB-Wax column (Phenomenex, Torrance, CA, USA). The GC oven was programmed from 80–180 at 15°C/min, following a delay of 1 min, and then to 200 at 3°C/min. Samples were injected splitlessly, with helium as carrier gas at 1 ml/min. Mostly, only the titer of the major pheromone component, (*Z*)-11-hexadecenal (Z11-16:Ald; Roelofs et al., 1974), was measured, as all *de novo* fatty acids and derived compounds are influenced by sugar feeding (Foster and Johnson, 2010). However, ratios of Z11-16:Ald: (*Z*)-9-tetradecenal (Z9-14:Ald), together capable of eliciting male flight (Vetter and Baker, 1983), were measured for 5-d-old virgins, to preclude the possibility that differences in male responses were caused by changes in female blend ratios. The identities of the two pheromone components was confirmed by comparison of their retention times and mass spectra with those of synthetic standards available in the laboratory.

Pheromone was collected from two 4-d-old virgins (either sugar- or water-fed) in a 200 ml glass cylinder with inlet and exhaust ports. Activated charcoal-purified air was pushed through the cylinder at 160 ml/min, and volatiles were collected on 150 mg of Tenax GC (60–80 mesh; Alltech Assoc., Deerfield, IL, USA) packed between two plugs of glass wool in a Pasteur pipette. The adsorbent was conditioned at 220°C for at least 1 h under nitrogen flow. Pheromone was collected for three periods: 0–2 h, 2–4 h, and 4–6 h of the scotophase. After collection, 50 ng of E11-14:Ald were placed on the glass wool, and chemicals eluted with 1.3 ml of *n*-hexane. The solvent was evaporated to 20 µl using nitrogen, and pheromone determined by GC-MS.

The MS was operated in the selected-ion mode, recording *m/z* 192 (E11-14:Ald and Z9-14:Ald) and 220 (Z11-16:Ald). Pheromone quantity or ratio was determined by integration of peak areas, with *m/z* intensities calibrated by standard solutions. In the text and figures, pheromone quantity is the amount of Z11-16:Ald.

Mating Frequencies and Times The propensity of sugar- and water-fed females (virgin and mated) to mate was determined in confined conditions. A single female and male (virgin, 1-2-d-old) were placed in a 150 ml container (Solo Cup Co., Urbana, IL, USA) 1 h before the onset of the scotophase. Pairs were observed every 15 min, over the next 9 h or until all had copulated. The time that copulation commenced was recorded.

Male Responses to Females The ability of sugar- or water-fed females (virgin or mated) to elicit male upwind flight was tested in a wind tunnel. Just prior to the scotophase,

females were placed individually in 30 ml containers (Solo Cup Co.), with nylon mesh lids and bases. A 0.45 m diam× 1.3 m long cylindrical wind tunnel, constructed from clear acrylic, was used. Smoothed air was pushed through the tunnel, by a fan, at 30 cm/sec. The tunnel floor was a 20 cm-wide white laminate sheet, with random 7.5 cm diam black circles for visual orientation, and was illuminated to 2 lux by six overhead, voltage-regulated tungsten lamps. An exhaust fan removed pheromone from the room.

Insects were tested at two times: from 0.5–2.5 h (early) and from 3–5 h (mid) of the scotophase. These periods were used because, peak sexual activity occurs at mid-scotophase (Heath et al., 1991), and because of the potential mating benefit for females by attracting males earlier in the scotophase. We tested 22 to 41 females for each treatment, alternating sugar- and water-fed females. This allowed us to reduce any problem of female variability, as well as compare proportions of females that elicited a full response (touch) from at least one male. We tested 3 males to each female, roughly over a 5 min period. Female *H. virescens* call continuously for multiple periods, each of up to an hour, throughout the scotophase (Ramaswamy, 1990). Therefore, we likely tested a female either when she was calling or not calling.

A female was placed on a 15-cm high stand, at the upwind end of the tunnel, so that the mesh ends of the container were in line with the airflow. Females acclimated for 3 min in the tunnel, before a male was released at the downwind end. The response of a male was recorded as: (1) whether or not it fanned wings ("activation") within 3 min of being in the tunnel, (2) whether or not it exhibited sustained flight upwind, orienting to a female ("upwind flight"), and (3) whether or not it contacted the female's container ("touch").

Statistical Analyses Experiments were conducted as paired blocks of sugar- and water-fed females, of a particular age and mating status. For pheromone analyses, sugar- and water-fed females, of a given age and photoperiodic time, were compared by ANOVA, after first confirming normality and heteroscedasity. For the bioassays, the various proportions (copulation, flight responses, females that elicited at least one male to "touch") of sugar- and water-fed females were compared using likelihood ratio *chi-square* tests. Mean times of copulation commencement were compared by ANOVA, after confirming normality and heteroscedasity.

Results

Pheromone Analyses Sugar- and water-fed 1-d-old virgins had similar pheromone titers (beginning $F_{1,26}=0.82$, $P=0.37$; mid $F_{1,20}=1.53$, $P=0.23$; end $F_{1,22}=1.74$, $P=0.20$) at each of the three scotophase times (Fig. 1a). Sugar- and water-fed 3-d-old virgins had similar titers at mid ($F_{1,27}=0.30$, $P=0.59$) and end scotophase ($F_{1,30}=1.80$, $P=0.19$), but sugar-fed females had greater ($F_{1,32}=9.95$, $P=0.004$) titers than water-fed ones at the beginning (Fig. 1b). Five-d-old sugar-fed virgins had greater pheromone titers at each of the three periods throughout the scotophase (beginning $F_{1,28}=5.11$, $P=0.032$; mid $F_{1,26}=23.9$, $P<0.001$; end $F_{1,32}=4.27$, $P=0.047$) than did water-fed females (Fig. 1c). Sugar-fed mated females had greater titers than water-fed ones, at the beginning ($F_{1,27}=4.35$, $P=0.047$) and middle ($F_{1,31}=4.93$, $P=0.034$), but not the end ($F_{1,28}=0.81$; $P=0.35$), of the scotophase. Overall, regardless of age and feeding status, titers increased from beginning to mid, and declined by the end of scotophase.

There was no difference ($F_{1,33}=1.37$; $P=0.25$) in mean ratio of Z11-16:Ald: Z9-14:Ald produced by 5-d-old sugar- (mean=100: 4.22±0.49; $N=17$) and water-fed (mean=100: 5.03±0.48; $N=18$) virgins.

Pheromone amount released by 4-d-old virgins was greater for sugar- than water-fed females, for each period (0–2 h $F_{1,18}=6.27$, $P=0.022$; 2–4 h $F_{1,19}=4.45$, $P=0.048$; 235 4–6 h $F_{1,16}=7.76$; $P=0.013$) (Fig. 2). Pheromone amount released, increased through the scotophase for both types of females.

Mating Frequencies and Times For 3-d-old virgin ($\chi^2=1.30$, $P=0.26$), 5-d-old virgin ($\chi^2=0.93$, $P=0.33$) and mated ($\chi^2=2.24$, $P=0.14$) females, there were no differences between the respective proportions of sugar- and water-fed females that mated (Fig. 3). The mean time that copulation commenced was similar for sugar- and water-fed females for 3-d-old virgins, 5-d-old virgins and mated females, with most copulations occurring between the hour preceding onset and the second hour of scotophase (Fig. 3).

Male Responses to Caged Females Activation (early $\chi^2=2.15$, $P=0.34$; mid $\chi^2=0.20$, $P=0.65$), upwind flight (early $\chi^2=1.25$, $P=0.54$; mid $\chi^2=0.12$, $P=0.73$); and touch (early $\chi^2=1.06$, $P=0.59$; mid $\chi^2=0.26$, $P=0.61$) responses of males were similar to sugar- and water-fed 3-d-old virgins, at both periods of the scotophase (Fig. 4a). Very high, and similar, proportions of sugar- or water-fed females elicited touch from at least one male at both early ($\chi^2=0.17$, $P=0.68$) and mid ($\chi^2=1.46$, $P=0.23$) scotophase (Fig. 4a).

Greater proportions of males activated (early $\chi^2=19.5$ $P<0.001$; mid $\chi^2=14.9$, $P<0.001$), flew upwind (early $\chi^2=18.6$ $P<0.001$; mid $\chi^2=29.9$, $P<0.001$); and touched (early $\chi^2=13.5$ $P<0.001$; mid $\chi^2=25.9$, $P<0.001$) cages containing 5-d-old sugar-fed virgins than cages containing 5-d-old water-fed virgins, at both time periods (Fig. 4b). The proportion of females that elicited touch from at least one

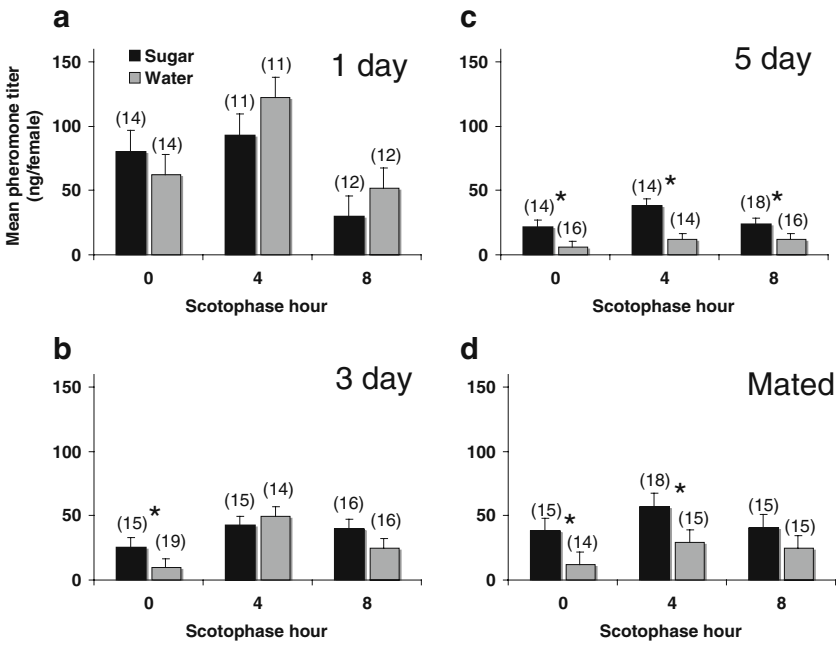


Fig. 1 Mean sex pheromone titers, at different scotophase times, of female *Heliothis virescens* fed on either 10% sucrose or water: **a** 1-d-old virgins, **b** 3-d-old virgins, **c** 5-d-old virgins, and **d** 3-d-old mated females. An asterisk above a pair of titers shows means that are different at $P<0.05$ (ANOVA). Ns are in parentheses

male was greater for sugar- than water-fed females, at both early ($\chi^2=8.35$, $P=0.004$) and mid ($\chi^2=15.5$, $P<0.001$) scotophase (Fig. 4b).

Sugar-fed, mated females elicited greater upwind flight (early $\chi^2=10.5$, $P=0.001$; mid $\chi^2=6.03$, $P=0.011$); and touch ($\chi^2=9.84$, $P=0.002$; mid $\chi^2=8.55$, $P=0.004$) responses from males than did water-fed, mated females, at both times. Activation was similar ($\chi^2=0.33$, $P=0.56$) to both female types at early, but greater ($\chi^2=7.41$, $P=0.007$) to sugar-fed females at mid-scotophase. The proportions of cages containing mated females touched by at least one male were similar ($\chi^2=0.26$, $P=0.61$) for both types (sugar- or water-fed) at early, and greater ($\chi^2=4.53$, $P=0.033$) for sugar-fed females at mid-scotophase.

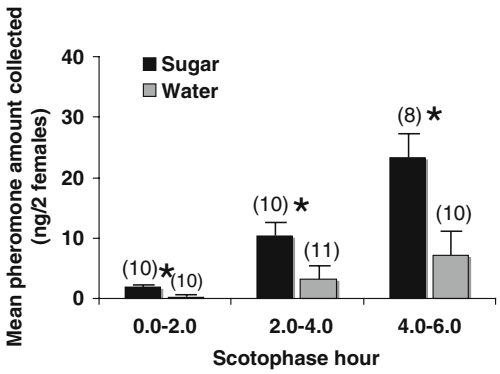


Fig. 2 Mean amount of sex pheromone released by 4-d-old virgin *Heliothis virescens* females, fed on either 10% sucrose or water, collected at different periods of the scotophase. An asterisk above a pair of titers shows means that are different at $P<0.05$ (ANOVA). Ns are in parentheses

Discussion

Honest signals for mate assessment must convey information to the receiver about the quality of a signaler, thereby enabling reliable differentiation of high quality signalers from low quality ones (Johansson and Jones, 2007). Signal honesty is thought to be maintained by increasing signal cost to signalers that can least afford it (Zahavi, 1977; Grafen, 1990). Here, we demonstrated that pheromone production and release by *H. virescens* sugar-replete females was greater than that by sugar-stressed females, and that greater proportions of males responded to the sugar-replete females than to the stressed ones. Our data do not allow us to distinguish between whether this quantitative difference is a function of different amounts released, or different intermittencies between release (calling), although the pheromone titer and male response data suggest the former is more likely. This quantitative difference in pheromone signal is related to female quality, with sugar-replete female *H. virescens* producing many more eggs than sugar-stressed ones (Ramaswamy et al., 1997). Thus, males benefit by distinguishing between females based on pheromone quantity. Moreover, sugar-replete females are likely to benefit from releasing more pheromone by increasing their chance of obtaining mates, particularly by competing more successfully for males earlier in the scotophase. As mating delays cause decreased fecundity in moths (e.g., Jiménez-Pérez and Wang, 2003), the benefit of mating earlier can be substantial for both sexes. Increased likelihood of further mating is important for mated females as, in polyandrous species such as *H.*

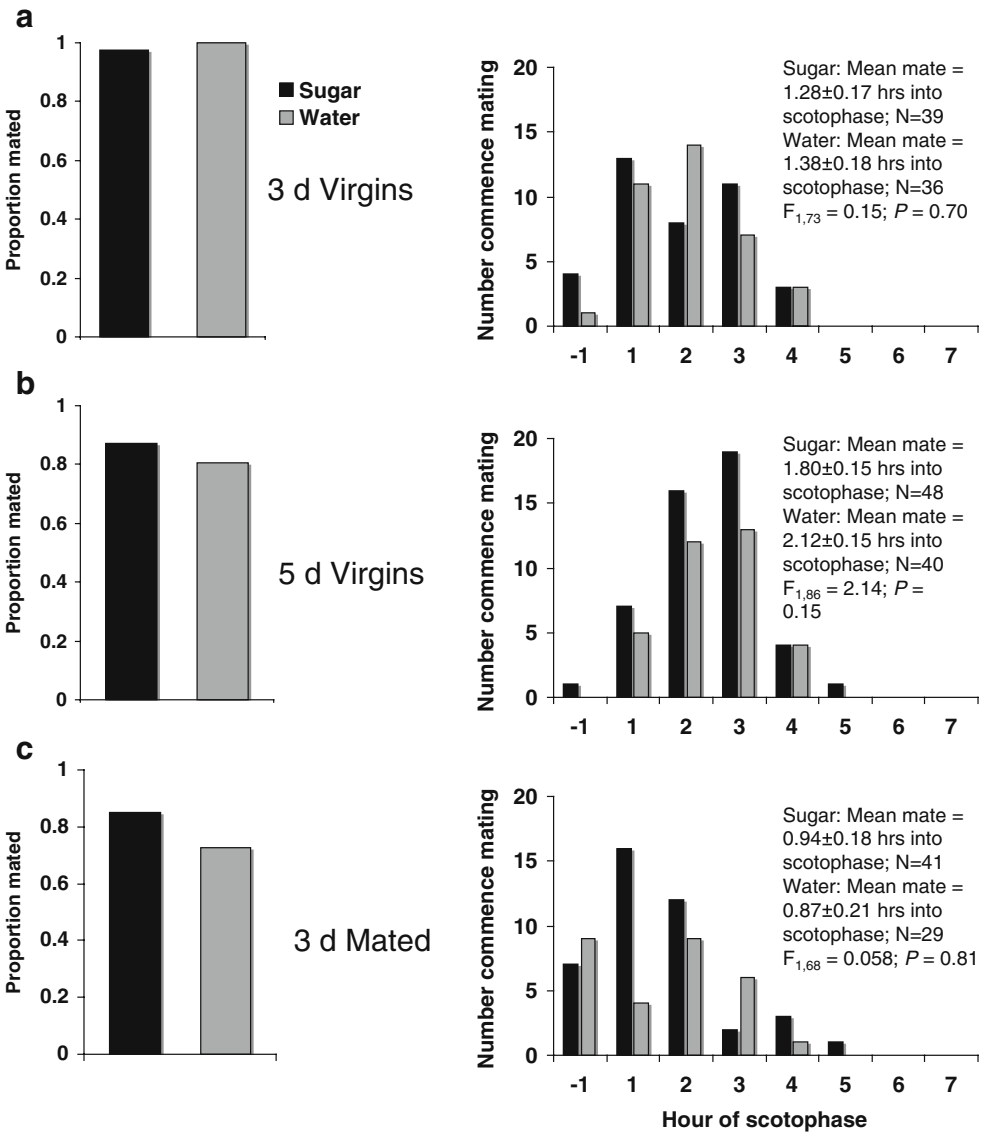


Fig. 3 Proportions and periodicities of *Heliothis virescens* copulations. Females were fed on either 10% sucrose or water: **a** 3-d-old virgins, **b** 5-d-old virgins, **c** 3-d-old mated females. There were no differences in proportions of, or mean times commencing, copulation between sugar- and water-fed females for each female category

virescens (Lamunyon, 2000), multiple mating causes greater fecundity (Torres-Vila et al., 2004). Therefore, the sex pheromone produced by female *H. virescens* is an honest signal for male assessment of a female's quality.

The relationship between pheromone quantity and fecundity is robust because pheromone and egg production share a common metabolite: adult sugar (O'Brien et al. 2000; Foster, 2009). We found no differences in pheromone quantity or male responses, with feeding status of young virgins, because these females, regardless of feeding status, have high HTC (Foster, 2009; Foster and Johnson, 2010); only when sugar-stressed (from mating, prolonged starvation) is the effect on pheromone manifest.

The possibility of male mate assessment based on female-produced moth sex pheromones has been proposed previously (Allison and Cardé, 2006, 2008) for the almond moth. Age-related changes in component ratios and female fecundity, led the authors to propose that males could assess female age and reproductive value through pheromone ratios (Allison and Cardé, 2008). However, almond moth females are considered mostly monandrous (McNamara et al., 2008) and, therefore, age-related mate selection would be effective only if high proportions of old females were unmated, which is unlikely. Males may also be capable of assessing the mating status of females through differences in female chemical signals (Thomas, 2011). For monandrous moth species, such as the almond moth, this is somewhat trivial, as sex pheromone production is probably permanently shut down in mated females (e.g., Foster, 1993). In contrast, in polyandrous species such as *H. virescens* (Lamunyon, 2000), sex pheromone production continues after mating, as shown in Fig. 1. In *H. virescens*, the effect of mating on pheromone production is governed largely by feeding status (HTC), with sugar-replete mated females having fairly similar pheromone titers to comparable virgins (Foster, 2009). Thus, males may be unable to

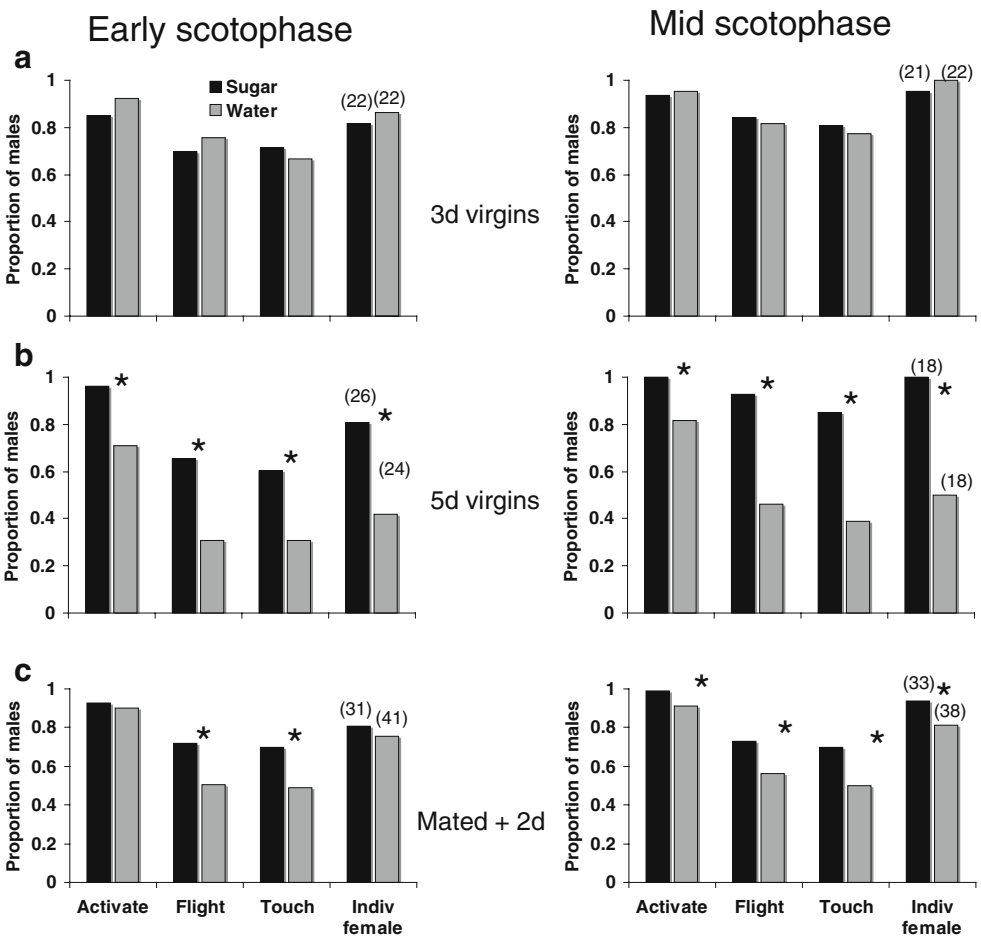


Fig. 4 Flight responses of male *Heliothis virescens* to individual females fed on 10% sucrose or water. **a** 3-d-old virgin, **b** 5-d-old virgin, **c** 3-d-old mated females. Trials were run at early (0.5–2.5 h after onset) and mid-scotophase (3–5 h after onset). Activate=wing fanning; Flight=sustained, oriented upwind flight; Touch=touched the container housing a female. The proportions of individual (Indiv) females that elicited at least one male to Touch are given. An asterisk above a pair of responses, indicates proportions that are different at $P<0.05$ (likelihood ratio chi-square tests). The numbers of females tested are given above the Indiv female bars (multiply by 3 for the total males flown per treatment)

differentiate sugar-replete mated females from virgins, on the basis of quantity of pheromone released alone.

Our behavioral studies showed that differential responses of males to the different female types were evident only over long distance, via pheromone quantity released. At close distance in *H. virescens*, hair pencil compounds are thought to mediate acceptance of males by females (Hillier and Vickers, 2004). Our confined experiment showed that female receptivity to copulation was unaffected by sugar stress, both in terms of mating proportions and latency to mate. Both bioassays were no-choice, because we felt this represented the natural situation better, in which isolated females release pheromone that distant males either respond to or not, rather than males assessing pheromone plumes of different females in close proximity to each other. Allison and Cardé (2008) also concluded that a no-choice situation was probably more natural for testing male mate assessment in the almond moth.

Previous work (Foster, 2009; Foster and Johnson, 2010) examined the effect of sugar stress at peak pheromone production in *H. virescens*. In this paper, we found that sugar stress impacts pheromone production and release throughout the scotophase. The patterns of production and release were similar for both female types, regardless of sugar stress, with peak release occurring after peak pheromone titer at mid-scotophase. While a similar relationship between peak titer and release has been found in other moths (e.g., Heath et al., 1991; Bäckman et al., 1997), our bioassays showed that most females attract and copulate with males much earlier in the scotophase than the peak release time. This suggests that female *H. virescens* produce and release pheromone in a martingale strategy, with increasing quantities released, the longer the female remains unmated through the scotophase, thereby increasing the chance of eventually attracting a male. This suggests that pheromone production may actually be a cost to a female, especially as after mating its production and release typically ceases (i.e., is conserved), at least for the remainder of the sexually active period (Raina, 1989; Foster, 1993). Pheromone production in female moths has been regarded as of little cost (Svensson, 1996), because small amounts of carbon are invested in pheromone compared to in eggs. Moreover, as both pheromone and egg production are adversely affected when females are sugar stressed, it is possible that pheromone production has a marginal cost, that impacts increasingly on fitness as a female's sugar resources are depleted. This marginal cost may be the increased signal cost, to signalers that can least

afford it, that maintains signal honesty (Zahavi, 1977; Grafen, 1990).

Further work is needed to test whether this phenomenon of male mate assessment through female pheromone quantity, based on female sugar resources, influences mate selection in the field, as well as expanding this work to other species of moths so as to establish whether it is a widespread phenomenon.

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Secondary Metabolites Released by The Burying Beetle *Nicrophorus vespilloides*: Chemical Analyses and Possible Ecological Functions

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Abstract Burying beetles of the genus *Nicrophorus* localize small vertebrate carcasses by emitted volatiles. The carcass that serves as reproduction and breeding site is buried in the soil by the beetles. Biparental care for offspring includes both preservation of the carrion and its preparation as diet and nursery. Buried carcasses show no signs of microbial decay, and those experimentally treated with *Nicrophorus* secretions are known to grow fewer bacteria and fungi. In order to investigate the chemical composition of these secretions, we used GC-MS for analysis of methanolic extracts of anal and oral secretions released by adult *N. vespilloides*. Furthermore, we analyzed the headspace of adult *N. vespilloides* by SPME-GC-MS and searched for compounds with known antimicrobial activity. We identified 34 compounds in the headspace, and anal and oral secretions, 26 of which occurred consistently. We discuss the ecological relevance of these compounds with respect to both their antimicrobial activity and ecological relevance.

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Introduction

Members of the coleopteran taxon Silphidae are commonly referred to as carrion beetles. Currently, the most prominent genus, *Nicrophorus*[1] (subfamily Nicrophorinae), comprises 68 species of burying or sexton beetles (Sikes, 2008), which are unique in their behavior of searching for small vertebrate carcasses, preferably of small rodents or birds, that are subsequently buried to be used as a reproduction site. This behavior was investigated in the pioneering study by Pukowski (1933) who provided the first detailed description of *Nicrophorus* ecology. She described the remarkable biparental care of this taxon for the offspring, which involves *i*) providing the offspring with intestinal content during the first 4–5 d of larval development, *ii*) defending larvae against predators, and *iii*) immediate repair of a destroyed brood chamber. In contrast, silphids outside the genus *Nicrophorus* do not bury or prepare a carcass. Their larvae develop externally by feeding on the carcass and maggots (Sikes, 2008).

[1] The genus was first described as *Nicrophorus* by Fabricius in 1775. It appears that the same name was used by Fabricius several times subsequently. By contrast, the etymologically more meaningful name *Necrophorus* (gr.: 'corpse carrier') describes a habit characteristic for this genus. Nevertheless, assuming *Nicrophorus* to be an incorrect transliteration, a change to *Necrophorus* would lead to a violation of the priority rule (Herman, 1964). Thus, the name *Necrophorus* must no longer be considered valid (Herman, 1964; Peck and Miller, 1982). Despite this, the latter spelling was still predominant in literature until the early 1990s.

Members of the genus *Nicrophorus* are able to locate a deceased animal within 1 d after its death, being attracted from a distance of up to several km (Kalinová et al., 2009). Carrion is located by chemoreceptors on the antennal club. Recent investigations have demonstrated that both *N. vespilloides* and *N. vespillo* are lured by several sulfur-containing organic volatiles, above all dimethylsulfide (DMS), dimethyldisulfide (DMDS), and trimethyltrisulfide (DMTS) (Kalinová et al., 2009). When a carcass has been located by a burying beetle, it is first checked for its size and properties (i.e., degree of decomposition). After acceptance of a carcass, the beetle defends this resource against intra- and interspecific competitors. The carcass is subsequently buried by a single *Nicrophorus* sp. pair that first removes the underlying soil and then moves the carrion down a slope into a cave. The spherical bottom of this brood chamber is termed 'crypt'. Hairs or feathers of the carrion are mechanically removed, and a 'brood ball' is formed. According to Pukowski (1933), the latter is impregnated with the beetle's feces and anal secretion. Larval development is accelerated and complete within seven days.

During the past three decades, research mainly has focused on aspects of *Nicrophorus* behavioral and reproductive ecology (Eggert et al., 1998; Scott, 1998; Meierhofer et al., 1999; Müller et al., 2007), along with the question of how phoretic mites (Mesostigmata: Parasitidae) such as *Poecilochirus carabi*, *P. necrophori*, and *P. subterraneus* affect *Nicrophorus* biology (Schwarz and Müller, 1992; Schwarz and Koulianos, 1998; Grossman and Smith 2008). By contrast, chemical ecology has barely been pursued for silphids in the past, although chemical signaling in beetles (Francke and Dettner, 2005) and chemical defense of insects (Laurent et al., 2005) have been major areas of research for more than six decades.

The few studies devoted to chemical ecology of Silphidae include analysis of the anal defensive spray of the red-lined carrion beetle *Necrodes surinamensis* (Eisner et al., 1986), the neutral fraction of which consists of three terpene alcohols: lavendulol, and two cyclopentanoids, (−)-α- and (−)-β-necrodol (Roach et al., 1990). From anal secretions of *Silpha americana* (Meinwald et al., 1985) and *S. novaboracensis*, several defensive steroids (pregnane derivatives) have been identified (Meinwald et al., 1987). More recent work on nest mate recognition (Müller et al., 2003) by *Nicrophorus* has addressed the role of polyunsaturated cuticular hydrocarbon patterns for discrimination between breeding partners and conspecific intruders (Steiger et al., 2007, 2008, 2009; Scott et al., 2008).

The process of burying has been shown to prevent infestation of a carcass by dipteran larvae (Suzuki, 2000). Furthermore, a carcass buried by *Nicrophorus* sp. shows hardly any sign of microbial decay, although it is completely buried in the soil (Pukowski, 1933). Burying beetles regularly come into close contact with high loads of carrion-associated gram-positive and gram-negative aerobic and (facultatively) anaerobic bacteria (Solter et al., 1989; Berdela et al., 1994). Consequently, burying beetles must avoid uncontrolled bacterial and fungal decomposition of the carrion (Scott, 1998). A digestive phospholipase A_2 (PLA_2) is present in the oral secretion of the North American species *N. marginatus*, but absent in the midgut. It has been hypothesized that this enzyme contributes to preservation of the carcass by attacking bacterial membranes (Rana et al., 1997). In a study on *N. quadripunctatus*, Suzuki (2001) demonstrated the importance of parental pre-treatment that effectively reduces fungal growth on carcasses. Observations by Rozen et al. (2008) led to the conclusion that *N. vespilloides*, whenever possible, tends to avoid breeding in carcasses that display advanced putrefaction. If impossible, extended parental care before and after larval hatching is applied that limits the negative effects of carcass decay induced by microorganisms.

Studies of the anal and oral fluids of seven North American *Nicrophorus* species have revealed that oral secretions from *N. marginatus*, *N. obscurus*, *N. americanus*, *N. tomentosus*, and *N. orbicollis* show antimicrobial activity, whereas the anal secretion is antimicrobially active only in the latter two species (Hoback et al., 2004). The lack of antimicrobial activity in the *N. pustulatus* fluids is attributed to the brood-parasitic behavior of this species, which uses carcasses that have already been pre-treated with antimicrobial fluids by other *Nicrophori.* However, the lack of antimicrobials in *N. carolinus* fluids has not been explained satisfactorily and recently was reconsidered (Jacques et al., 2009). So far, the anal and oral secretions of *Nicrophorus* are known to display pronase and heat stability (Hoback et al., 2004), however, their antimicrobially active compounds are unknown.

Here, we chemically analyzed extracts of anal and oral secretions of *N. vespilloides* by GC-MS. Furthermore, we studied the headspace of adult beetles by SPME-GC-MS.

Methods and Materials

Origin of Beetles Parasite-free *N. vespilloides* males and females were provided by Josef K. Müller and Wolf Haberer (Department of Evolutionary Biology and Ecology, Faculty of Biology, University of Freiburg, Germany). The method of obtaining a culture of 'foster parents' free of mites and nematodes has been described in detail by Eggert et al. (1998). Groups of up to 6 adults of the same sex were kept in transparent plastic containers (100×100×65 mm; Famos-Westmark, Lennestadt-Elspe, Germany), two-thirds filled with soil (Fruhstorfer Erde®, type T, structure 1b; distributed by Hawita Group GmbH, Vechta, Germany), in

a 20°C constant temperature room under a 16/8 h photoperiod. Each beetle was fed one freshly decapitated mealworm twice a week.

Rearing of Beetles Reproduction in *Nicrophorus* species is induced easily by providing a male-female pair with a suitable mouse carcass. Mice which have been deep-frozen immediately after killing were obtained from the Department of Medical Microbiology, University of Giessen, Germany. Approximately 4 h before darkness, when beetles typically start searching for carrion, a mouse which had been thawed for 8 h was placed on top of the soil surface, and a pair of beetles was added. Parents were removed 5 d after hatching of the larvae. Containers were transferred to darkness after 3rd instars appeared on top of the soil surface for pupation.

Sampling To minimize possible influences of inconsistent diet on the composition of the secretions analyzed, only sexually mature, non-breeding beetles that had been fed exclusively on mealworms (*Tenebrio molitor*) were used. Mealworms were reared in the same 20°C constant temperature room under a 16/8 h photoperiod in transparent plastic containers (Geo Medium, Ferplast S.p.A., Vicenza, Italy) and fed exclusively on oat flakes (C. Hahne Mühlenwerke GmbH & Co. KG, Löhne, Germany). All beetles analyzed were at least 3 wk-old. The day the young beetle left the pupal chamber was considered d 1 of the adult stage. Only larvae experienced a mouse-based diet during biparental care, whereas adults were fed mealworms in order to avoid contamination of the analyzed secretions with carcass-derived compounds.

Headspace Sampling by SPME For analysis of VOC's emitted by adult *N. vespilloides*, one individual beetle was transferred carefully into a 20 ml amber GC vial with screw cap and septum (CS—Chromatographie Service, Langerwehe, Germany) by using flexible forceps. This vial was placed in the autosampler of a CTC Combi Pal unit that was subsequently agitated for 3 min at room temperature, thus inducing the beetle to regurgitate or to secrete anal fluid. The headspace subsequently was absorbed for 3 min onto a solid-phase micro-extraction fiber (SPME, coating polydimethylsiloxane [PDMS], Supelco, Bellefonte, PA, USA), then desorbed for 3.5 min, and finally analyzed by gas chromatography-mass spectrometry (GC-MS).

Sampling of Anal and Oral Secretions For analysis of anal and oral fluids, the respective secretions from 5–15 beetles were sampled into a 150 µl glass insert placed in a 1.5 ml HPLC vial with screw cap (CS—Chromatographie Service, Langerwehe, Germany). Samples of each sex were collected separately. Oral fluid was released when gently placing the beetle's head into the wide opening of the insert, whereas anal fluid was obtained by gently pressing the beetle's abdominal tip into the opening of the insert. All vials were kept on crushed ice during sampling. Each sample was mixed with 50 µl of methanol, vortexed for 3 min, and placed back on ice. After sedimentation, the supernatant was transferred into the glass insert of an amber GC vial (CS—Chromatographie Service, Langerwehe, Germany) and analyzed by direct-inlet GC/MS. Animals were transferred back into the container immediately after sampling, and each beetle was provided with a freshly decapitated mealworm.

GC/MS Analysis—General Analyses were performed on a Varian CP 3800 gas chromatograph with a CTC Combi Pal unit coupled to a Varian Saturn 2000 EI mass spectrometer (Varian, Darmstadt, Germany). The system was equipped with a Varian VF-XMS column/precolumn combination (30 m×0.25 mm, df=0.25 µm). Helium was used as carrier gas at a flow rate of 1.0 ml/min. EI mass spectra were recorded with an ionization voltage of 70 eV in the mass range of m/z 35–450. Total analysis time was 30 min. Data analysis was performed by the Saturn GC/MS Workstation 5.52 software, including the 2006 NIST/EPA/NIH Mass Spectral Library. Tentative compound identifications by the NIST Library were verified by analyzing retention time and EI fragmentation patterns of commercial standard samples purchased from Sigma-Aldrich-Fluka (Steinheim, Germany). 2-Acetamido-2-deoxy-D-mannolactone was obtained from Carbosynth Ltd. (Compton, Berkshire, UK). Whenever possible, positional isomers such as isoquinoline vs. quinoline as well as *o*- and *m*- vs. *p*-cresol were included to unambiguously confirm our identification. Only those peaks displaying a signal-to-noise ratio≥3 were considered for interpretation. All reference substances (*trans*-geranylacetone, phenol, *o*-, *m*-, and *p*-cresol, indole, benzaldehyde, phenylethyl alcohol [= 2-phenylethanol], isobutyric acid, *n*-butyric acid, isovaleric [3-methylbutyric] acid, (±)-2-methylbutyric acid, *n*-valeric acid, *N,N'*-dimethyl urea, benzoic acid, 2-piperidone [= δ-valerolactam], *N*-methylpiperidine, 1-methyl-2-pyrrolidinone, caprylic acid [= *n*-octanoic acid], pelargonic acid [= *n*-nonanoic acid], 2-phenylacetic acid, quinoline, isoquinoline, 3-phenylpropionic acid, 2-acetamido-2-deoxy-D-mannolactone, capric acid [= *n*-decanoic acid], *m*- and *p*-hydroxybenzyl alcohol, 2-, 3- [= skatole], 5-, 6-, and 7-methylindole, *p*-hydroxybenzoic acid methyl ester [= methylparaben], 2-indolinone [= oxindole], *N*-(2-phenylethyl)-acetamide, *m*- and *p*-hydroxybenzoic acid, *m*-and *p*-hydroxyphenylacetic acid, lauric acid [= *n*-dodecanoic acid], methylmyristate, myristic acid, methylpalmitate, palmitic acid, methylstearate, oleic acid, and stearic acid) and organic solvents were of GC/MS or LC/MS quality (purity usually≥99%). Water for SPME headspace GC/MS was

purified by a Millipore Q system (Millipore, Schwalbach, Germany) providing a conductance of 18.2 MΩ/cm.

GC/MS Analysis of Headspace Samples Conditions were as follows: injector, initial temperature 50°C, ramped at 200° C/min, final temperature 270°C, hold for 10 min, splitless mode; column oven, initial temperature 50°C, ramped at 10°C/min, final temperature 250°C, hold for 6 min. Reference standards (1 mg of solids, 1 µl of liquids, or dilutions thereof) were suspended in 10 ml of water and sampled at 60°C instead of room temperature.

GC/MS Analysis of Anal and Oral Fluids Conditions were as follows: injector, initial temperature 50°C, hold for 0.1 min, ramped at 200°C/min, final temperature 250°C, hold for 10 min, splitless mode; column oven, initial temperature 50° C, hold for 2.6 min, ramped at 10°C/min, final temperature 250°C, hold for 7.4 min. Reference standards (1 mg of solids, 1 µl of liquids) were dissolved in 4 ml of methanol; if required, diluted in methanol and analyzed.

Results

Both oral and anal secretions of *N. vespilloides* are of dark brown color and exhibit a characteristic odor. The bouquet of the latter is pungently putrefactive, suggesting the dominance of phenolic (phenol, *p*-cresol) and amine (indole) components. The scent of the former, however, is less penetrating, suggesting the presence of fatty acids. These differences were confirmed by the acidity of the respective samples: native anal secretions had pH 9, whereas oral secretions had pH 5–6, thus confirming data from Hoback et al. (2004).

The volume of secretion released by each beetle varied considerably. Thus, a number of minor components, although detectable in trace amounts, could not be considered for analysis in each sample. All metabolites in our study were identified by comparison of EI-MS fragmentation patterns, and were numbered consecutively according to their order of appearance in this Results section.

SPME-GC/MS Analysis—Headspace Analysis of Adult N. vespilloides (Fig. 1, Table 1) Headspace samples were obtained from 14 individual beetles (7 of each sex). As living beetles are assumed to simultaneously secrete both anal and oral fluids into the GC vial, volatiles found in the headspace may originate from either secretion. Ethyl-4-methyl heptanoate, previously described as a male-produced pheromone of *N. vespilloides* (Haberer et al., 2008), was not detected in our samples. No qualitative differences were observed between total ion current

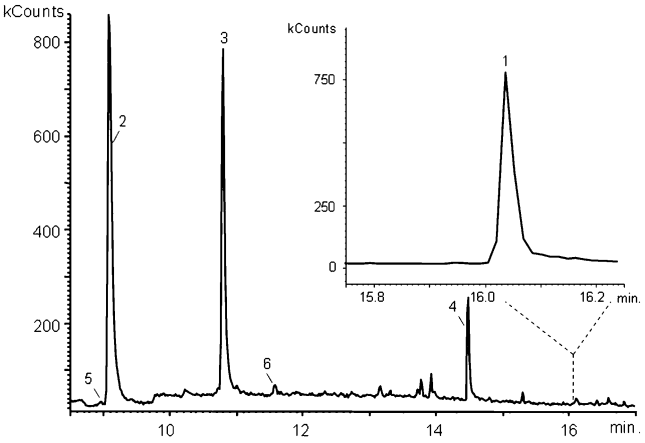


Fig. 1 GC-MS total ion current chromatogram of *Nicrophorus vespilloides* headspace. The sample was collected from one living female beetle. The position of (*E*)-geranylacetone (**1**) produced by calling sexually mature males is indicated in the insert

chromatograms of male and female beetles, except for the presence or absence of (*E*)-6,10-dimethyl-5,9-undecadien-2-one ('*trans*-geranylacetone': **1**), which is released by sexually mature males (Haberer et al., 2008).

Three major compounds were detected in the headspace of adults: phenol (**2**), *p*-cresol (**3**), and indole (**4**). In addition, small amounts of benzaldehyde (**5**) and 2-phenylethyl alcohol (**6**) were frequently detected. The presence of (**2**) and (**4**) in anal secretions of *N. vespilloides* recently has been reported by Haberer et al. (2010).

GC/MS Analysis of the Anal Secretion of N. vespilloides (Fig. 2, Table 1) Four samples, 2 of each sex, were analyzed. Samples were pooled from 5 and 10 males, as well as from 10 and 15 females, respectively. The pattern of low-molecular metabolites found in anal secretions was remarkably diverse, consisting of short-, medium-, and long-chain fatty acids, phenols, aromatic carboxylic acids, aldehydes, alcohols, amines, and amides. The following metabolites, listed in ascending order by retention time, were identified unambiguously (Fig. 2): isobutyric (2-methylpropionic, **7**), *n*-butyric (**8**), isovaleric (= 3-methylbutyric, **9**), (±)-2-methylbutyric acid (**10**),[2] *n*-valeric acid (**11**), benzaldehyde (**5**), phenol (**2**), *p*-cresol (**3**), *N,N'*-dimethyl urea (**12**), 2-phenylethyl alcohol (**6**), benzoic acid (**13**), 2-piperidone (= δ-valerolactam, **14**), pelargonic acid (= *n*-nonanoic acid, **15**), 2-phenylacetic acid (**16**), quinoline (**17**), indole (**4**), 3-phenylpropionic acid (**18**), 2-acetamido-2-deoxy-D-mannolactone (**19**), *m*- or *p*-hydroxybenzyl alcohol (**20**), skatole (= 3-methylindole, **21**), *p*-hydroxybenzoic acid methyl ester (= methylparaben, **22**), 2-indolinone (= oxindole, **23**), *N*-(2-phenylethyl)-acetamide (**24**),

[2] (+)- and (−)-2-methylbutyric acid could not be separated under the GC conditions applied.

Table 1 Possible contribution of individual metabolites detected to the carcass-preserving effect of *Nicrophorus vespilloides* secretions

Metabolite (**No.**) name		Origin	Produced by male(♂)/female(♀)	Consistency of occurrence[*1] [%]	Presumed biological significance for *N. vespilloides* (References)
(**1**)	*(E)*-geranylacetone	(?)	♂	57 (♂)	male-produced, significance unknown (Haberer et al., 2008)
(**2**)	phenol	H/A	♂/♀	100 (H) 100 (A)	insect repellent, antibacterial and antifungal disinfectant (Duffey et al., 1977; Goddard and McCue, 2001)
(**3**)	*p*-cresol	H/A	♂/♀	86 (H) 100 (A)	invertebate repellent (Duffield et al., 1977); antibacterial and antifungal disinfectant (Lüllmann et al., 2006)
(**4**)	indole	H/A/O	♂/♀	100 (H) 100 (A) 100 (O)	insect repellent (Duffield et al., 1977); antibacterial, antifungal (Devi et al., 2010)
(**5**)	benzaldehyde	H/A	♂/♀	21 (H) 25 (A)	deterrent to invertebrate predators (Duffey et al., 1977; Kelley and Schilling, 1998)
(**6**)	2-phenylethyl alcohol	H/A	♂/♀	50 (H) 50 (A)	broad-spectrum antibacterial (Ali et al., 2001)
(**7**)	isobutyric acid	A	♀	25	antimicrobial (Stratford and Eklund, 2003); contribution to the spread- and penetration-enhancing ability of long-chain fatty acids (Roach, et al., 1990); repellent to insect predators (Dettner and Reissenweber, 1991)
(**8**)	*n*-butyric acid	A/O	♂/♀	75(A) 100(O)	
(**9**)	isovaleric acid	A/O	♂/♀	100(A)	
(**10**)	(±)-2-methylbutyric acid	A/O	♂/♀	100(O)	
(**11**)	*n*-valeric acid	A	♀	100(A) 100(O)	
				25	
(**12**)	*N,N'*-dimethyl urea	A	♀	25	hydroxyl radical-scavenging (Fischer and Hamburger, 1980)
(**13**)	benzoic acid	A/O	♂/♀	100	E 210: antibacterial, antifungal (Duffey et al., 1977; Roncadori et al., 1985)
(**14**)	2-piperidone	A/O	♂/♀	100	(?)[*2]
(**15**)	pelargonic acid	A/O	♂/♀	100	antimicrobial (Stratford and Eklund, 2003)
(**16**)	phenylacetic acid	A/O	♂/♀	100	antibacterial, antifungal (do Nascimento et al., 1996; Hwang et al., 2001; Kim et al., 2004; Mendonça et al., 2009)
(**17**)	quinoline	A/O	♂/♀	100	antiseptic (Frerichs et al., 1930)
(**18**)	3-phenyl-propionic acid	A/O	♂/♀	50 (A) 100 (O)	antibacterial, antifungal (Dettner and Schwinger, 1980; Narayana et al., 2007)
(**19**)	2-acetamido-2-deoxy-D-mannolactone	A/O	♂/♀	50 (A) 100 (O)	(?)
(**20**)	*m*-/*p*-hydroxy-benzyl alcohol	A	♀	25	antioxidant, radical-scavenging (Dhiman et al., 2009)
(**21**)	skatole	A	♀	25	repellent to vertebrate and invertebrate predators (Blum et al., 1973; Duffield et al., 1977)
(**22**)	*p*-hydroxy-benzoic acid methyl ester	A	♀	25	E 218: broadly antimicrobial (Schildknecht, 1970; Classen and Dettner, 1983; Stratford and Eklund, 2003)
(**23**)	2-indolinone	A	♂/♀	50	(?)
(**24**)	*N*-(2-phenyl-ethyl)acetamide	A	♂/♀	75	(?)[*3]
(**25**)	*p*-hydroxy-benzoic acid	A	♀	25	antibacterial (Huberman et al., 2007); anti-*Candida albicans* (Ata et al., 2011)
(**26**)	*p*-hydroxy-phenylacetic acid	A	♀	25	antibacterial (Huberman et al., 2007)
(**27**)	methylmyristate	A/O	♂/♀	100	spread- and penetration-enhancement of antimicrobial and repellent metabolites (Roach et al., 1990); insect repellent (Henderson and Jeanne, 1989)
(**28**)	myristic acid	A/O	♂/♀	100	
(**29**)	methylpalmitate	A/O	♂/♀	100	
(**30**)	palmitic acid	A/O	♂/♀	100	
(**31**)	methylstearate	A/O	♂/♀	100	

Table 1 (continued)

Metabolite (**No.**) name		Origin	Produced by male(♂)/female(♀)	Consistency of occurrence[*1] [%]	Presumed biological significance for *N. vespilloides* (References)
(**32**)	oleic acid	A/O	♂/♀	100	insect repellent (Eisner et al., 1996; Dani et al., 1996); 'necromone' (Rollo et al., 1994)
(**33**)	stearic acid	A/O	♂/♀	100	see (**28**)–(**32**); 'necromone' (Rollo et al., 1994)
(**34**)	caprylic acid	O	♂/♀	100	antimicrobial (Stratford and Eklund, 2003)

n. d.—not detected, *i. e.*, metabolite not present or signal-to-noise-ratio below the 3:1 threshold; H—headspace (N=14=100%); A—anal secretion (N=4=100%); O—oral secretion (N=2=100%); (?)—biological significance for *N. vespilloides* remains unresolved. [*1]—a metabolite is defined consistent if it is present in ≥ 50% of the samples analyzed. [*2]—recently isolated as an end product of the L-amino acid oxidase escaping present in the ink of sea hares (*Aplysia californica*: Kamio et al., 2009), [*3]—frequently isolated as a biologically inactive by-product of antibiotic fermentations (Asolkar et al., 2002; Maskey et al., 2003).

p-hydroxybenzoic acid (**25**), *p*-hydroxyphenylacetic acid (**26**), methylmyristate (**27**), myristic acid (**28**), methylpalmitate (**29**), palmitic acid (**30**), methylstearate (**31**), oleic acid (**32**), and stearic acid (**33**).

GC/MS Analysis of the Oral Secretion of N. vespilloides (data not shown) Two samples, 1 of each sex, were analyzed. Samples were pooled from 5 males and 15 females, respectively. The chromatogram was dominated by (**28**), (**30**), (**32**), and (**33**) whereas (**9**), (**10**), caprylic (*n*-octanoic acid, **34**), (**13**), (**14**), and (**15**) were present in minor amounts.

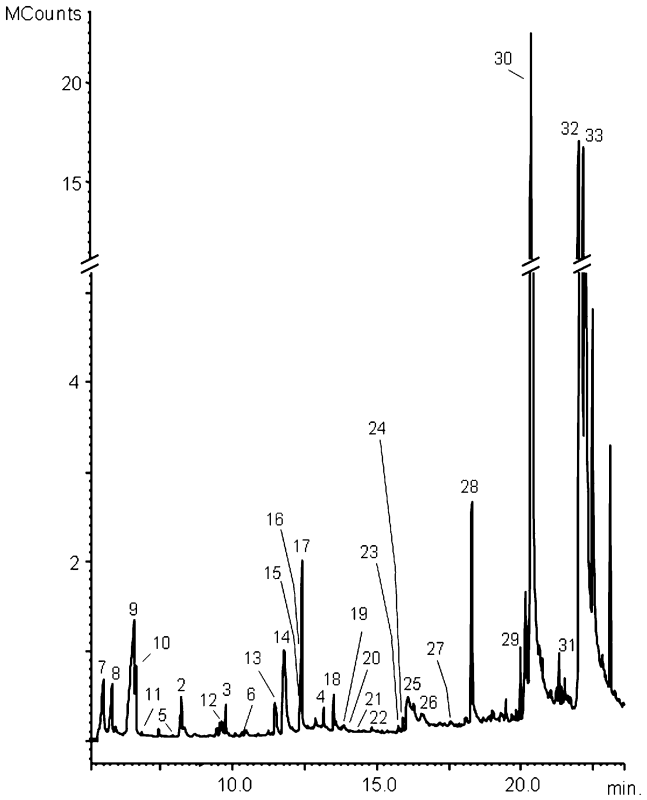


Fig. 2 GC-MS total ion current chromatogram of *Nicrophorus vespilloides* anal secretion. The sample was collected from 15 female beetles

Neutral (**19**) and amine components, (**17**) and (**4**), were detected in traces, only.

Discussion

Metabolic Origin of Compounds Detected in Nicrophorus Secretions The majority of the metabolites detected originate from amino acid catabolism. The detected short-chain volatile fatty acids (VFA's) have been previously reported as derived from amino acid precursors. A study by Elsden and Hilton (1978) on *Clostridium* sp. revealed that strains that were able to oxidize valine to isobutyric acid (**7**) also oxidized leucine to isovaleric (= 3-methylbutyric) acid (**9**) and isoleucine to 2-methylbutyric (**10**) acid. Depending on the species tested, threonine served as a precursor of propionic, *n*-butyric (**8**), or *n*-valeric acid (**11**). Further studies on the end products of fermentation of aromatic amino acids by clostridia revealed that phenylalanine served as the precursor of either phenylacetic or phenylpropionic acid (Elsden et al., 1976), whereas tyrosine yielded *p*-hydroxyphenylacetic acid (**26**), phenol (**2**), and *p*-cresol (**3**). Tyrosine and phenylalanine also have been discussed as precursors of phenol (**2**), and *p*-cresol (**3**), benzoic acid (**13**), and *p*-hydroxybenzoic acid. Deamination of tryptophan leads to the formation of skatole (**21**) at low pH values, whereas high pH values favor the production of indole (**4**) (Le et al., 2005; Swann et al., 2010). 2-Piperidone (= δ-valerolactam, **14**) previously has been shown to originate from lysine degradation *via* cadaverine, 1-piperideine, and subsequent cyclization of 5-aminopentanoic acid (Callery and Geelhaar 1984). The C_{14}-, C_{16}-, and C_{18}-fatty acids and their corresponding methyl esters (**27**–**33**) may originate from the activity of lipolytic enzymes such as phospholipase A_2 (Uscian et al., 1995; Rana et al., 1997; Nor Aliza and Stanley 1998).

The structures of the metabolites found indicate that a highly diverse, as yet uninvestigated community of microbial symbionts is likely to contribute to their biosynthesis in *N.*

Table 2 Distribution of *Nicrophorus vespilloides* secondary metabolites amongst insects and other taxa

Metabolite No.	Name	Producing Species (Order, Family)	Origin of secretion	References
(1)	*(E)*-geranylacetone	*Atta sexdens sexdens* (Hymenoptera, Formicidae)	abdominal sex-attracting secretion (♀)	(Francelino et al., 2006)
(2)	phenol	*Costelytra zealandica* (Coleoptera, Scarabaeidae) *Melolontha hippocastani*, *M. melolontha* (Coleoptera, Scarabaeidae)	abdominal sex-attracting secretion (♀) whole body extracts of ♀, sex pheromone for ♂	(Henzell and Lowe, 1970; Ruther et al., 2001; Ruther et al., 2002; Zarbin, et al., 2007)
		Phyllophaga cuyabana (Coleoptera, Scarabaeidae)	sex pheromone from abdominal gland (♀)	
(3)	*p*-cresol	*Phyllophaga cuyabana* (Coleoptera, Scarabaeidae) *Pycopsyche scabripennis* (Trichoptera, Limnephilidae)	sex pheromone from abdominal gland (♀) secretion from defensive glands	(Zarbin et al., 2007; Duffield et al., 1977)
			defensive secretion from abdominal glands	
(4)	indole	*Pycopsyche scabripennis* (Trichoptera, Limnephilidae) *Kheper bonellii* (Coleoptera, Scarabaeidae)	defensive secretion from abdominal glands abdominal sex-attracting secretion (♂)	(Duffield et al., 1977; Burger et al., 2008a)
(5)	benzaldehyde	*Cicindella* sp., *Megacephala carolina* (Coleoptera, Cicindelidae)	defensive secretion from pygidial glands	(Kelley and Schilling, 1998)
(6)	phenylethyl alcohol	*Trachypachus gibbsii* (Coleoptera, Trachypachidae)	defensive secretion from abdominal glands	(Attygalle et al., 2004)
(7)	isobutyric acid	*Trachypachus gibbsii* (*Coleoptera, Trachypachidae*) *Omalinum rugatum*, *O. caesum*, *O. rivulare*, *Lathrimaeum atrocephalum*, *L. unicolor*	defensive secretion from abdominal glands	(Attygalle et al., 2004; Dettner and Reissenweber, 1991)
(8)	*n*-butyric acid	*Phyllodrepa floralis* (Coleoptera, Staphylinidae, Omaliinae)	defensive secretion from abdominal glands	(Dettner and Reissenweber, 1991)
(9)	isovaleric acid	*Omalinum rugatum*, *O. caesum*, *O. rivulare*, *E. stramineum*, *E. sorbi*, *E. abdominale*, *E. ophthalmicum*, *E. limbatum*, *E. torquatum*, *E. minutum*, *E. florale*, *Phleonomus pusillus*	defensive secretion from abdominal glands	(Dettner and Reissenweber, 1991; Burger, et al., 2002; Burger et al., 2008a)
		Kheper subaeneus, *Kheper bonellii* (Coleoptera, Scarabaeidae)	abdominal sex-attracting secretion (♂)	
(10)	(±)-2-methyl-butyric acid	*Trachypachus slevinii* (Coleoptera, Trachypachidae) *Trachypachus gibbsii* (Coleoptera, Trachypachidae)	defensive secretion from abdominal glands	(Attygalle et al., 2004; Dettner and Reissenweber, 1991)
		Omalinum rugatum, *O. caesum*, *O. rivulare*, *Lathrimaeum atrocephalum*, *L. unicolor*, *Eusphalerum marshami*, *E. stramineum*, *E. sorbi*, *E. abdominale*, E. *ophthalmicum*, *E. limbatum*, *E. torquatum*, *E. minutum*, *E. florale*, *Phleonomus pusillus* (Coleoptera, Staphylinidae, Omaliinae); *Proteinus brachypterus*, *P. atomarius*, *P. macropterus* (Coleoptera, Staphylinidae, Proteininae)		
(11)	*n*-valeric acid	*Asaphidion flavipes*, *Bembidion quatriguttatum* (Coleoptera, Carabidae)	defensive secretion from abdominal glands	(Schildknecht, 1970)
(12)	*N,N'*-dimethyl urea	*Panthera tigris* (Carnivora, Felidae) *Sus domestica* (Articodactyla, Suidae)	territorial marking fluid of ♂ Bengal tigers decaying carcasses	(Burger et al., 2008b; Dekeirsschieter, et al., 2009; Swann et al., 2010)
(13)	benzoic acid	*Dytiscus marginalis*, *Abagus bipustulatus*, *A. paludosus* (Coleoptera, Dytiscidae)	defensive secretion from abdominal glands	(Schildknecht et al., 1962; Schildknecht and Weis 1962; Classen and Dettner 1983)
(14)	2-piperidone	Sea hare (*Aplysia californica*: Anaspidea, Aplysiidae) *Cornitermes weberi* (Isoptera, Nasutitermitinae)	defensive secretion (ink) against predators frontal gland secretion of workers	(Kamio et al., 2009; Blum et al., 1982; Cork, 1994)
		Cochliomyia hominivorax (Diptera, Calliphoridae)	extracts of wound fluid from sheep wounds infested with larvae	
(15)	pelargonic acid	*Trachypachus slevinii* (Coleoptera, Trachypachidae)	defensive secretion from abdominal glands	(Attygalle et al., 2004)

Table 2 (continued)

Metabolite No.	Name	Producing Species (Order, Family)	Origin of secretion	References
(16)	2-phenylacetic acid	*Atta sexdens rubropilosa*, *A. cephalotes*, *Acromyrmex octospinosus* (Hymenoptera, Formicidae)	secretion from metathoracic (metapleural) glands	(do Nascimento et al., 1996; Mendonça et al., 2009
(17)	quinoline	*Oreophotes peruana* (Phasmatodea, Diapheromeridae)	defensive secretion from thoracic glands	(Eisner et al., 1997)
(18)	3-phenyl-propionic acid	*Hydatis seminiger* (Coleoptera, Dytiscidae, Dytiscinae)	defensive secretion from pygidial glands	(Dettner and Schwinger 1980)
(19)	2-acetamido-2-deoxy-D-mannolactone	not yet isolated from insects		
(20)	*m*-/*p*-hydroxy-benzyl alcohol	not yet isolated from insects		
(21)	skatole	*Kheper lamarcki* *Kh nigroaeneus* *Kh subaeneus* *Kh bonellii* (all Coleoptera, Scarabaeidae) *Pycopsyche scabripennis* (Trichoptera, Limnephillidae)	abdominal sex-attracting secretion (♂) defensive secretion from abdominal glands	(Burger and Petersen, 2002; Burger et al., 2002; Burger et al., 2008a; Duffield et al., 1977)
(22)	*p*-hydroxy-benzoic acid methyl ester	*Dytiscus marginalis*, *Cybister lateralimarginalis, Acilius sulcatus, Graphoderes cinereus* (Coleoptera, Dytiscidae, Dytiscinae); *Rhantus exoleus*, *Colymbetes fuscus*, *Copelatus ruficollis*, *Agabus sturmi*, *A. bipustulatus*, *Ilybius fenestratus* (Coleoptera, Dytiscidae, Colymbetinae)	defensive secretion from pygidial glands	(Schildknecht, 1970)
(23)	2-indolinone	*Cochliomyia hominivorax* (Diptera, Calliphoridae)	extracts of wound fluid from sheep wounds infested with larvae	(Cork, 1994)
(24)	*N*-(2-phenyl-ethyl)acetamide	not yet isolated from insects		
(25)	*p*-hydroxy-benzoic acid	*Graphoderes cinereus* (Coleoptera, Dytiscidae, Dytiscinae); *Rhantus exoleus*, *Colymbetes fuscus* (Coleoptera, Dytiscidae, Colymbetinae)	defensive secretion from pygidial glands	(Schildknecht, 1970)
(26)	*p*-hydroxy-phenylacetic acid	*Hydroporus dorsalis* (Coleoptera, Dytiscidae, Hydroporinae); *Noterus clavicornis* (Coleoptera, Dytiscidae, Noterinae)	defensive secretion from pygidial glands	(Dettner and Schwinger 1980)
(27)	methylmyristate	*Polistes fuscatus* (Hymenoptera, Vespidae)	ant repellent from van der Vecht's gland	(Henderson and Jeanne 1989)
(28)	myristic acid	*Ceraeochrysa smithi* (Neuroptera, Chrysopidae)	defensive egg stalk fluid	(Eisner et al., 1996)
(29)	methylpalmitate	*Pieris rapae crucivora* (Lepidoptera, Pieridae)	exocrine secretions	(Honda and Kawatoko 1982)
(30)	palmitic acid	*Necrodes surinamensis* (Coleoptera, Silphidae)	defensive secretion from rectal gland	(Roach et al., 1990)
(31)	methylstearate	*Pieris rapae crucivora* (Lepidoptera, Pieridae)	exocrine secretions	(Honda and Kawatoko 1982)
(32)	oleic acid	*Ceraeochrysa smithi* (Neuroptera, Chrysopidae)	defensive egg stalk fluid	(Eisner et al., 1996)
(33)	stearic acid	*Ceraeochrysa smithi* (Neuroptera, Chrysopidae) *Necrodes surinamensis* (Coleoptera, Silphidae)	defensive egg stalk fluid defensive secretion from rectal gland	(Eisner et al., 1996; Roach et al., 1990)
(34)	caprylic acid	*Necrodes surinamensis* (Coleoptera, Silphidae) *Omalinum rugatum*, *O. caesum*, *O. rivulare*, *Lathrimaeum atrocephalum*, *L. unicolor*, *Anthophagus angusticollis*, *Eusphalerum marshami* (Coleoptera, Staphylinidae, Omaliinae)	defensive secretion from rectal gland defensive secretion from abdominal glands	(Roach et al., 1990; Dettner and Reissenweber, 1991)

vespilloides. So far, *N. tomentosus* is the only species of burying beetles whose microbial flora in the midgut, hindgut, and hemolymph has been partly characterized (Solter et al., 1989; Berdela et al., 1994).

Putative Ecological Function of Metabolites Detected in Nicrophorus Secretions The high microbial load and the high viscosity of the secretions prevented us from testing the antimicrobial activity of our small, volatile samples, which could not be effectively sterilized. Possible contributions of the 34 individual metabolites identified in this study to the carcass-preserving effect *N. vespilloides* are compiled in Table 1. Most of the substances found have been reported previously as products of putrefaction (Dekeirsschieter et al., 2009; Swann et al., 2010); however, many also exhibit a pronounced anti-putrefactive, antimicrobial activity. Given that a breeding couple of *N. vespilloides* regularly and repeatedly impregnates the carcass in the crypt (Pukowski, 1933), we hypothesize that burying beetles are able to positively regulate the microbial colonization of carrion. Most of the primary and secondary metabolites found in the headspace and secretions of adult *N. vespilloides* might serve this ecological function.

Occurrence of Compounds in Other Taxa and Their Dependence on the Physiological State of the Beetle Overall, most of the primary and secondary metabolites we found are widespread amongst invertebrate taxa (Table 2): A total of 68 species are known in the taxon *Nicrophorus*, but hydrolytic enzymes such as phospholipase A_2 have so far been shown only in *N. marginatus* (Rana et al., 1997). The presence of such an enzyme in anal and oral secretions of *N. vespilloides* might provide free fatty acids, as detected in our samples. To further elucidate the pattern of secondary metabolites responsible for preservation of carcasses, anal and oral secretions could be extracted by less polar GC-compatible solvents and analyzed with or without further derivatization. Furthermore, anal and oral secretions should be investigated by state-of-the-art HPLC/MS-MS methods.

The composition of anal and oral secretion might vary with the physiological state of the beetle. Studies (Müller et al., 2003; Steiger et al., 2007, 2008, 2009) have shown that the cuticular hydrocarbon profile of *N. vespolloides* varies between breeding and non-breeding mates. Similarly, the monoterpenoid (*E*)-methylgeranate is emitted only by breeding *N. vespolloides,* not by non-breeding beetles (Haberer et al., 2010). Furthermore, the pattern of metabolites present in breeding *N. vespilloides* could change in the course of time during parental care. As diet previously has been shown to influence the antimicrobial properties of oral secretions in *N. carolinus* and *N. marginatus* (Jacques et al., 2009), the metabolite pattern of beetles feeding on a rodent carcass might be different from that of beetles feeding on freshly decapitated mealworms. Finally, studies on the microbial flora found in secretions of *Nicrophorus vespilloides* should be considered as a future point of interest in order to gain further insight into the biosynthesis and decomposition of small metabolites.

In summary, we report for the first time a detailed analysis of secondary metabolites secreted by *N. vespilloides.* These metabolites could contribute both to defense against competitors and to chemical preservation of carcasses used for reproduction and as a diet for offspring. So far, our knowledge of secondary metabolites of *Nicrophorus* sp. remains fragmentary and additional research is needed for basic understanding of chemical carcass preservation by burying beetles.

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Two Genera of Aulacoscelinae Beetles Reflexively Bleed Azoxyglycosides Found in Their Host Cycads

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Abstract Aulacoscelinae beetles have an ancient relationship with cycads (Cycadophyta: Zamiaceae), which contain highly toxic azoxyglycoside (AZG) compounds. How these "primitive" leaf beetles deal with such host-derived compounds remains largely unknown. Collections were made of adult *Aulacoscelis appendiculata* from *Zamia* cf. *elegantissima* in Panama, *A. vogti* from *Dioon edule* in Mexico, and *Janbechynea paradoxa* from *Zamia boliviana* in Bolivia. Total AZG levels were quantified in both cycad leaves and adult beetles by high performance liquid chromatography (HPLC). On average, cycad leaves contained between 0.5–0.8% AZG (frozen weight, FW), while adult beetles feeding on the same leaves contained even higher levels of the compounds (average 0.9–1.5% FW). High AZG levels were isolated from reflex bleeding secreted at the leg joints when beetles were disturbed. Nuclear magnetic resonance and mass spectroscopy identified two AZGs, cycasin and macrozamin, in the reflex bleeding; this is the first account of potentially plant-derived compounds in secretions of the Aulacoscelinae. These data as well as the basal phylogenetic position of the Aulacoscelinae suggest that sequestration of plant secondary metabolites appeared early in leaf beetle evolution.

Key Words Aulacoscelinae · *Aulacoscelis* · Azoxyglycosides · Chrysomelidae · Coleoptera · *Dioon* · *Janbechynea* · Reflex bleeding · *Zamia* · Zamiaceae

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Introduction

One of the least understood plant-insect interactions is that between cycads and aposematic herbivorous beetles in the subfamily Aulacoscelinae (Chrysomelidae *sensu lato*), a basal lineage of leaf beetles closely associated with the neotropical cycad family Zamiaceae (Cycadophyta: Gymnosperm) (Windsor et al., 1999; Santiago-Blay, 2004). Cycads contain toxic azoxyglycosides (AZGs); herbivore defense compounds that have the common aglycone, methylazoxymethanol (MAM) (Bowers and Larin, 1989). The two most common cycad AZGs, cycasin and macrozamin (the β-glucoside and β-primeveroside of MAM, respectively), are found in the seeds and leaves of all cycad genera (De Luca et al., 1980; Yagi, 2004). These glycosylated storage forms are non-toxic but upon herbivory, endogenous plant or gut-associated enzymes cleave AZGs to the aglycone, which readily reacts with nucleic acids resulting in potentially mutagenic changes (Kobayashi and Matsumoto, 1965; Laqueur and Spatz, 1968; Schneider et al., 2002). Sequestration of AZGs from the host plant has been observed in a number of cycad-specialist herbivores. The larvae of the hairstreak butterfly (*Eumaeus atala florida* Poey, Lycaenidae: Lepidoptera) and of the

arctiid moth (*Seirarctia echo* Abbot and Smith, Arctiidae: Lepidoptera) feed on the leaves of *Zamia floridana* A.DC. In both herbivore species, the sequestered cycasin is retained in the adult stage where it is concentrated in the wings and abdomen (Teas, 1967; Rothschild et al., 1986). High β-glycosidase activity in the gut of the arctiid moth suggests that free MAM is re-glycosylated to restore cycasin after sequestration. Cycasin in *E. atala* deters ant predators and, probably, vertebrate predators as well (Bowers and Larin, 1989). The pollinating weevil of *Zamia furfuraceae* L. fil., *Rhopalotria mollis* Sharp (Belidae: Coleoptera), also sequesters cycasin from the male cones (Schneider et al., 2002).

Reflex bleeding refers to hemolymph-related secretions that are often associated with defensive posturing in many insect species. Aulacosceline adults exhibit a reflex bleeding mechanism similar to that observed in the Meloideae (Coleoptera) where upon disturbance of the insect, a droplet of hemolymph is exposed through the joints of the tibia and femur (Carrel and Eisner, 1974). The droplet is reabsorbed when the disturbance ceases. The purpose of this study was to determine whether AZG compounds are sequestered and secreted by cycad-feeding Aulacoscelinae leaf beetles.

Methods and Materials

Natural History Observations and Sample Collection Adult aulacosceline leaf beetles were collected from their respective cycad host plants by using a sweep net at three study sites: Huasteca Potosina, Mexico (May–September), Chagres National Park, Panama (May), and Potrerillos del Guenda, Bolivia (November) between 2008 to 2010. *Janbechynea paradoxa* Monrós and *Aulacoscelis appendiculata* Cox & Windsor suddenly appeared in large numbers as voracious feeders on the new leaves of *Zamia boliviana* (Brongn.) A.DC. in Bolivia and on *Zamia* cf. *elegantissima* Schutzman, Vovides and Adams in Panama, respectively. *Aulacoscelis vogtii* Monrós was observed in smaller numbers on *Dioon edule* Lindl. leaves during the rainy season in Mexico. Undamaged fully grown leaflets were collected and immediately frozen at −20°C until analysis. Adult beetles were removed from their host plants and the head, thorax, abdomen, and legs were manually separated using forceps. Three to four individuals of each species were pooled and frozen at −20°C for AZG analysis. Reflex bleeding was collected from live adults by gently holding the beetles with tweezers and transferring the droplets collected from the femur-tibia joints to filter paper by using capillary tubes. Beetle hemolymph was collected by centrifuging decapitated insects. The hemolymph and filter paper containing reflex bleeding were frozen at −20°C until extraction.

Azoxyglycoside Extraction and High Performance Liquid Chromatography Analysis Insect and leaf tissues were weighed and ground in 1 ml of chilled 70% ethanol. Homogenates were centrifuged at 13,000 rpm for 5 min, and the supernatant was split into subsamples; the first was used to measure basal levels of MAM and the second allowed measurement of AZGs through MAM equivalents. To measure AZG equivalents, the second subsample was diluted in sodium acetate buffer (pH 5.0) containing 0.27 U (enzyme unit) of β−glucosidase (Sigma) and incubated overnight in the dark at room temperature. Samples were syringe-filtered for HPLC analysis. Separation was achieved on a Cyano column (Varian Microsorb MV 100–5 CN, 150×4.6 mm) using a gradient from 75% methanol to 25% methanol in 10 min at a flow rate of 1.2 ml/min (Yagi, 2004). Peaks were monitored by a photodiode array detector at 215 nm. MAM standards were prepared by incubating commercially available MAM acetate (National Cancer Institute) in Tris–HCl buffer (final concentration 1 mg/ml, pH 8.0) with 36.9 U of esterase enzyme (Sigma) for 1 h at 25°C and used to generate a standard curve.

NMR and MS of Reflex Bleeding To identify the AZGs present in the reflex bleeding of *Janbechynea paradoxa*, four samples were pooled (approx. 10 μl), extracted in 50% methanol, and purified by HPLC. The concentrated sample was dried in a rotary evaporator and re-suspended in deuterated water for proton nuclear magnetic resonance analysis using a Bruker 300 MHz and accurate mass was obtained by sodium electrospray ionization ($[M+Na]^+$) MS/MS using an Agilent LS/MSD TOF mass spectrometer.

Results

Natural History Observations Little is known about the lifecycle of Aulacoscelinae beetles, except that the adults appear suddenly and for a brief period feed voraciously on the younger leaves of their cycad host plants during the onset of the rainy season (Windsor et al., 1999). Their long, sharp mandibles pierce the epidermis releasing plant juices that are then ingested. Mating occurs while the females are feeding.

Plant and Insect Azoxyglycoside Content The leaf AZG content of three Zamiaceae species ranged from 0.5–0.8% frozen weight (FW) on average (Table 1). *Dioon edule* showed the highest values (0.8%). For adult Aulacoscelinae beetles, the AZG content was higher, ranging from 0.9–1.5% FW. Trace amounts of AZGs were detected in the adult beetle head, thorax, abdomen, legs, and hemolymph.

Table 1 Azoxyglycoside content of adult Aulacoscelinae species and their respective host cycads

Insect Species	Fresh weight %±SD	*N*	Host Plant Species[b]	Fresh weight %±SD	*N*
Aulacoscelis appendiculata	1.49±0.77	4	*Zamia* cf. *elegantissima*	0.67±0.2	6
Aulacoscelis vogtii	1.12±1.04	4	*Dioon edule*	0.81±0.13	6
Janbechynea paradoxa	0.88±0.46	4	*Zamia boliviana*	0.52	2

[a] Whole body of adult insect. [b] Mature undamaged leaves

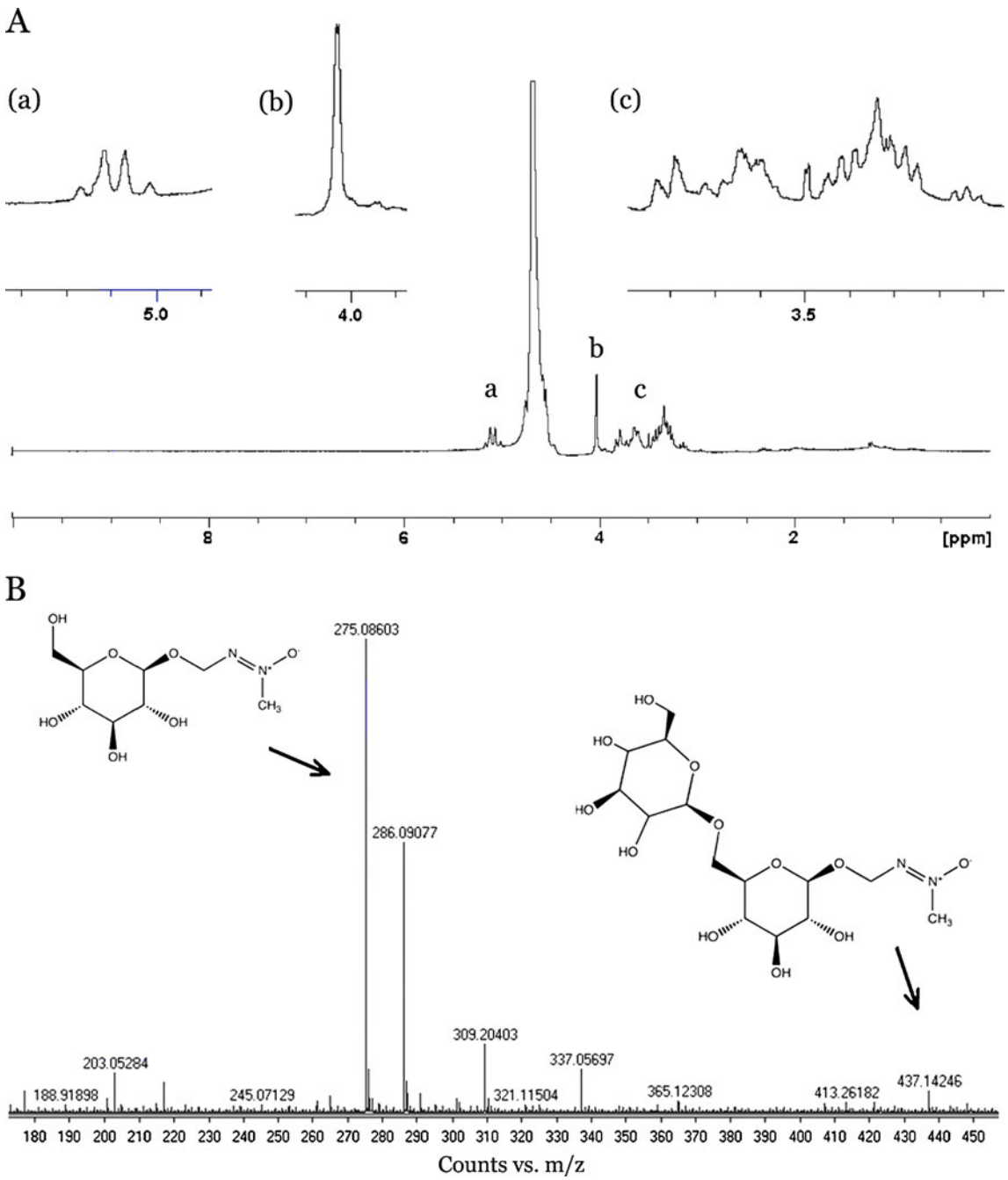


Fig. 1 Azoxyglycosides identified from the reflex bleeding of *Janbechynea paradoxa*. **A** ^{1}H-NMR spectrum of cycasin in deuterated water; *a*) anomeric CH, *b*) CH_3 and *c*) other CH, CH_2 and OH. **B** Accurate mass spectrometry of reflex bleeding; peaks corresponding to cycasin (275.096 m/z) and macrozamin (437.142 m/z) are identified

Azoxyglycosides in Adult Beetle Reflex Bleeding In response to disturbance, adult beetles secreted a droplet of reflex bleeding from the joint between the tibia and the femur. The color of the secretion ranged from clear to yellow and had a penetrating smell. The droplet was produced from any set of legs but usually was a localized response from the leg nearest the region of the body being held. Reflex bleeding (approx. 1.2 μl) was observed in all three Aulacoscelinae: *Aulacoscelis appendiculata, Aulacoscelis vogtii*, and *Janbechynea paradoxa.* Proton magnetic resonance spectra of secretions from *J. paradoxa* confirm that the reflex bleeding contained AZGs with diagnostic signals observed at 5.11, 4.03, and from 3.1 to 3.8 ppm (Fig. 1A). Mass spectroscopy identified a mixture of compounds with masses of 275.08, 286.09, 309.2, 337.06, and 437.14 *m/z*, indicating that the major component of the sample is cycasin (275.08603 *m/z*) with minor levels of macrozamin (437.14246 *m/z*) (Fig. 1B).

Discussion

Our observations suggest that toxic AZGs sequestered from the host plant may be part of the aulacosceline beetle defenses as they are exuded by the adult in response to disturbance (Fig. 1). Given the rarity of this class of compound in nature, it is likely that their abundance in insect herbivores reflects leaf chemistry as proposed by Schneider et al. (2002). Beetles were collected on specific plant species, and their sequestered AZG content is comparable to that of the host; however, in one of the field sites, Huasteca Potosina Mexico, another Zamiaceae species, *Ceratozamia mexicana,* grows near the *Dioon edule* population where insects were collected and might serve as an alternative AZG source for *Aulacoscelis vogtii.* The presence of both cycasin and macrozamin in the reflex bleeding suggests that these compounds are taken directly from the gut to the hemolymph through an active transport mechanism. A less likely possibility is that the sugar moiety is removed from AZG by glycosidases releasing toxic MAM that moves freely into the hemolymph, and then is re-glycosylated to form cycasin as proposed for *S. echo* caterpillars (Teas, 1967). Further research is being conducted to determine how Aucaloscelinae species accumulate these compounds and if they are capable of *in situ* synthesis.

As the strong smell of the secretion cannot be attributed to the AZGs, other plant- or insect-derived chemicals might also be part of the aulacosceline defense system. For example, AZGs are not the only compounds known to protect cycads against herbivores. The non-protein amino acid β-*N*-methylamino-L-alanine (BMAA) has the capacity to degenerate neural systems, and is found in several cycad tissues (Seawright et al., 1990). BMAA also could be part of the Aulacoscelinae defensive secretions, but this was not addressed in our study.

In an earlier review on defensive glands and secretions in the Chrysomelidae, Deroe and Pasteels (1982) lamented the lack of information available regarding Aulacoscelinae defense mechanisms. The authors proposed that for insects that do not have defensive glands associated with the surface of their elytra and pronotum as seen for other leaf beetle families, hemolymph-associated reflex bleeding may be an alternate defense mechanism. Here, we report that the Aulacoscelinae do not possess defensive glands in their elytra or pronotum but, in fact, reflexively bleed when disturbed. These secretions contain unusual AZG compounds also found in their host plants. The Aulacoscelinae is a sparse lineage of gymnosperm-associated herbivores, diverging alongside ancestors of the plant-feeding beetle superfamilies Chrysomeloidea and Cerambycoidea (Cox and Windsor, 1999). Putative Aulacoscelinae fossils date back to the Jurassic and earliest Cretaceous (Medvedev, 1968; Zhang, 2004). Therefore, the identification of plant-related secondary metabolites in Aulacoscelinae tissues and reflex bleeding opens the possibility that the ability to sequester plant-derived toxins may have appeared early in leaf beetle evolution, perhaps even predating the appearance of angiosperms.

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Identification of Semiochemicals Released by Cotton, *Gossypium hirsutum*, Upon Infestation by the Cotton Aphid, *Aphis gossypii*

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Abstract The cotton aphid, *Aphis gossypii* (Homoptera: Aphididae), is increasing in importance as a pest worldwide since the introduction of Bt-cotton, which controls lepidopteran but not homopteran pests. The chemical ecology of interactions between cotton, *Gossypium hirsutum* (Malvaceae), *A. gossypii*, and the predatory lacewing *Chrysoperla lucasina* (Neuroptera: Chrysopidae), was investigated with a view to providing new pest management strategies. Behavioral tests using a four-arm (Pettersson) olfactometer showed that alate *A. gossypii* spent significantly more time in the presence of odor from uninfested cotton seedlings compared to clean air, but significantly less time in the presence of odor from *A. gossypii* infested plants. *A. gossypii* also spent significantly more time in the presence of headspace samples of volatile organic compounds (VOCs) obtained from uninfested cotton seedlings, but significantly less time with those from *A. gossypii* infested plants. VOCs from uninfested and *A. gossypii* infested cotton seedlings were analyzed by gas chromatography (GC) and coupled GC-mass spectrometry (GC-MS), leading to the identification of (*Z*)-3-hexenyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), methyl salicylate, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), which were produced in larger amounts from *A. gossypii* infested plants compared to uninfested plants. In behavioral tests, *A. gossypii* spent significantly more time in the control (solvent) arms when presented with a synthetic blend of these four compounds, with and without the presence of VOCs from uninfested cotton. Coupled GC-electroantennogram (EAG) recordings with the lacewing *C. lucasina* showed significant antennal responses to VOCs from *A. gossypii* infested cotton, suggesting they have a role in indirect defense and indicating a likely behavioral role for these compounds for the predator as well as the aphid.

Key Words *Gossypium hirsutum* · *Aphis gossypii* · *Chrysoperla lucasina* · Cotton · Induced defense · Olfactometer · EAG · Repellency · (*Z*)-3-Hexenyl acetate · (*E*)-4,8-Dimethyl-1,3,7-nonatriene · Methyl salicylate · (*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene

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Introduction

Cotton, *Gossypium hirsutum* (Malvaceae), is a major fiber crop grown in over 100 countries. The earliest cultivation of cotton in the Americas occurred in Mexico 8,000 years ago (Wendel and Albert, 1992; Seelan et al., 1997), while for Asia, cotton cultivation was reported in India as far back as the Indus valley civilization, 7,000 years ago (Stein, 2001). The largest producers of cotton are China and India, with annual production of about 30 million bales and 24 million bales, respectively, in 2010 (http://www.cotton.org/econ/cropinfo/cropdata/rankings.cfm); most of this production is consumed by their own textile industries.

The primary route to the control of insect pests of cotton crops involves the use of synthetic chemical pesticides. To reduce reliance on pesticide usage, genetically modified cotton was introduced, which involved expression of genetic material associated with toxin production derived from *Bacillus thuringiensis* (Bt). After the introduction of Bt cotton, initial reports suggested that there was substantial reduction in the use of pesticides for control of bollworms (Qaim and De Janvry, 2005, Wu et al., 2008) without altering the status of sucking pests. However, recent reports show a surge in populations of sucking pests, including mirid bugs (Lu et al., 2010) and stink bugs (Willrich et al., 2004a, b, c; Siebert et al., 2005; Reay-Jones et al., 2010), with a steady increase in the use of pesticides (Anon., 2010). Several insecticides such as carbofuran, aldrin, and, more recently, thiamethoxam (Kharbouti and Allen, 2000) are reported to be effective. However, negative effects on non-targets in crop ecosystems and a perceived negative environmental impact have been reported, together with resistance to insecticides (Kerns and Gaylor, 1992; Herron et al., 2001; Herron and Wilson, 2010). A number of alternative pest control practices, such as habitat management (Landis et al., 2000), inundative release of predatory lacewings, *C. rufilabris* (Rosenheim and Wilhoit, 1993), and use of nucleopolyhedrovirus (Xia, 1997) have been tried in the past, but have failed to make much impact against the target due to poor efficacy, limited availability, high cost, and low adoptability.

The cotton aphid, *Aphis gossypii* Glover (Homoptera: Aphididae), is a major sucking pest on a global scale, having a wide host range and attacking many economically important crops (Kerns and Gaylor, 1992). Apart from direct damage, it is a vector of many viral diseases such as P (PRSV-P) and W (PRSV-W) strains of Papaya Ringspot Virus, Watermelon Mosaic Virus 2 (WMV-2), Cucumber Mosaic Virus (CMV), Celery Mosaic Virus (Francki et al., 1979), and Zucchini Yellow Mosaic Virus (ZYMV) (Blua and Perring, 1992). Direct damage and virus transmission are caused by the sucking behavior, mainly on the underside of leaf surfaces. Infested leaves often become cupped downwards and may appear wrinkled. Heavy infestations on some hosts may result in wilting, while young plants may have reduced or stunted growth. The aphids produce copious amounts of honeydew, which serves as a medium for sooty mould. This blackens the leaf and decreases photosynthetic activity (Elmer and Brawner, 1975). Adult *A. gossypii* generally are wingless, but a high population density induces the production of winged (alate) individuals that will migrate to new food sources.

To overcome problems associated with insecticide use in *A. gossypii* control, natural plant resistance, compatible with other more eco-friendly pest management practices (Biswas and Singh, 1998), has been investigated. Marker-assisted selection offers a means to increase the efficiency of incorporating aphid resistance in new plant varieties, but although some effort has been directed towards melon crops (Klingler et al., 2001), for cotton no progress has been reported. Furthermore, breeding approaches have been slow and difficult to achieve by traditional selection, and the availability of pest resistant cultivars to the farming community in India is unlikely in the foreseeable future. Another option is to exploit natural plant defense pathways, including the production of volatile semiochemicals (behavior-modifying chemicals) on insect herbivory (Bruce and Pickett, 2007). These volatile organic compounds (VOCs) attract parasitic and predatory insects that are natural enemies of the herbivores. They also may induce defense responses in neighboring plants (Pickett and Poppy, 2001). Although considerable attention has recently been directed towards plant/insect interactions in agricultural crops, relatively little has been done on the chemical ecology of cotton and associated sucking insect pests such as *A. gossypii*.

Currently, insecticides are the only available and widely used measure for controlling *A. gossypii*; because of the global rise in problems associated with sucking pests on cotton during recent years (Lu et al., 2010; Anon., 2010), more sustainable strategies are required. Lacewings (Neuroptera: Chrysopidae) are major aphid predators (Canard, 2001), and some widely distributed species such as green lacewings within the genus *Chrysopa* have been evaluated as biological control agents (Koczor et al., 2010). Here, we studied the chemical ecology of interactions between cotton and *A. gossypii* and, to provide evidence that *A. gossypii* induced defense can be detected at the next trophic level, we used adult predatory lacewings, *Chrysoperla lucasina* Lacroix (Neuroptera: Chrysopidae), as a model natural enemy.

Methods and Materials

Insect and Plant Material Aphids, *A. gossypii*, were reared on cotton, *G. hirsutum* cv. Linda, in an insectary (20°C; 16:8 h L:D regime) at Rothamsted Research, Harpenden,

Hertfordshire, UK. Cotton seedlings required for rearing *A. gossypii*, headspace collection and behavioral tests were grown under controlled conditions (25°C; 16:8 h L:D regime) in 15 modules or "cells" per standard seed tray, 2 seeds per cell. At the 2 weeks stage, trays were transferred into a growth room (25°C; 16:8 h L:D regime), where they were illuminated from above by fluorescent lights positioned 60 cm above bench height. Lacewings, *C. lucasina*, were obtained as adults from Koppert B.V., The Netherlands, and maintained in cages at 20°C, 16:8 h L:D, prior to use in coupled GC-electrophysiology.

VOC Collection Air entrainment (also referred to as dynamic headspace collection) was carried out following standard procedures (Webster et al., 2008) using 3-wk-old cotton plants. Seeds were planted singly in 13 cm plastic pots inside a growth chamber (25°C, 16:8 L:D). For each headspace collection, a seedling was enclosed in a glass vessel (22 cm high × 10 cm internal diam), open at the bottom, and with two collection ports at the top (one for inlet of air and the other for outlet). The bottom was closed without pressure around the plant stem, using two semicircular aluminum plates with a hole in the center to accommodate the stem. The plates were clipped to a flange on the open end of the glass vessel. Air, purified by passage through an activated charcoal filter (BDH, 10–14 mesh, 50 g), was pumped into the vessel through the inlet port at 700 ml/min (flow rate was controlled by a needle valve and measured by a flow meter). Air was drawn out at 600 ml/min through 50 mg Porapak Q (Alltech Associates, Carnforth, Lancashire, UK) in a 5 mm diam glass tube The differences in flow rates created a slight positive pressure to ensure that unfiltered air did not enter the system, thus removing the need for an airtight seal around the stem. All connections were made with polytetrafluoroethylene (PTFE) tubing (Alltech Associates) with brass ferrules and fittings (North London Valve, London, UK) and closed with PTFE tape (Gibbs and Dandy, Luton, UK). Glassware, metal plates, and other equipment were washed with aqueous Teepol detergent (Herts County Supplies, Herts, UK), acetone, and distilled water, and then baked overnight at 180°C. Porapak Q filled tubes were conditioned before use by washing with redistilled diethyl ether (1 ml) and heating inside an oven (132°C) under a stream of nitrogen for a minimum of 2 hr. Once conditioned, Porapak Q tubes were closed with Swagelok cap fittings and left to cool at ambient temperature prior to use. VOCs were collected on Porapak Q tubes inserted into the collection port on the top of the vessel and were subsequently eluted with 750 µl of freshly distilled diethyl ether.

VOCs were obtained from infested (100 *A. gossypii* per plant) and uninfested cotton seedlings. Aphids were released and allowed to settle for 1 hr before entrainments began. Plants were entrained for a total of 5 days, with the tubes being changed at 24 hr periods. Samples were combined prior to use in behavioral, electrophysiological, and chemical analytical studies, and were stored at −20°C until required for use. For electrophysiological studies with *C. lucasina*, the headspace sample of *A. gossypii* infested cotton was concentrated to *ca.* 50 µl under a gentle stream of nitrogen prior to use.

Four-Arm Olfactometer Bioassay Bioassays were conducted in a controlled environment room (22±2°C, 40% RH) fitted with an extraction fan. A Perspex olfactometer, having four glass arms and a central space connecting all the arms (10 cm diam, 14 cm arm long), was kept on a small stand. The central area of the top portion of the olfactometer contained a hole into which alate (winged) *A. gossypii* were introduced, and which was connected to a low pressure air pump. The olfactometer was set up in a cage (60 × 60 × 76 cm) comprising a steel frame covered with black cardboard paper to avoid external visual cues. Uniform illumination was provided by two fluorescent light tubes (70 W Luminux) positioned approximately 45 cm above the olfactometer. A charcoal-filtered airstream (200 ml/min) was supplied to the central space of the olfactometer using an electric pump. Adult winged aphids were collected from rearing cages in a separate insectary room and starved for 2 hr before each trial. Each aphid was exposed to the test samples for 16 min, and every 2 min the position of the olfactometer was rotated by 180°. The time spent by *A. gossypii* in the different arms of the olfactometer was recorded using a software programme (OLFA: F. Nazzi, Udine, Italy).

Several series of bioassays were conducted. Ten replicates were done for each sample. In the first series, the responses of *A. gossypii* to odor from *A. gossypii* infested and uninfested 3-wk-old cotton seedlings were studied, as were responses to VOCs obtained from the headspace above *A. gossypii* infested and uninfested seedlings. In the second series, the response was studied of *A. gossypii* to a synthetic blend of *A. gossypii* induced compounds (identification described later) [(*Z*)-3-hexenyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), methyl salicylate, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT)] and individual compounds, all at the levels found in the *A. gossypii* infested VOC sample. The doses used in the first and second series were equivalent to the amount emitted by one cotton seedling over 19.8 min. The blend also was tested in combination with the VOCs collected from uninfested cotton seedlings. For the third series, individual compounds were tested at a single dose (1 µg), either alone or in combination with the VOCs collected from uninfested cotton seedlings. In all series, the treatment sample (10 µl) was added to a filter paper, and the solvent was allowed to evaporate over 30 sec prior to placement in

a single arm, while the three control arms contained a piece of filter paper with 10 µl of control solvent (diethyl ether or hexane for VOC sample and synthetic blend, respectively). Treatments were offered to insects for the duration of each assay (16 min). Treatments were replaced with fresh material between replicates. Data were analyzed using a paired *t*-test (one tail) with statistical software (Genstat Version 11, VSN International Ltd.) after ensuring that data were normally distributed.

GC Analysis Samples of VOCs (4 µl) were analyzed on an Agilent 6890 GC equipped with a cool on-column injector, flame ionization detector (FID) and a non-polar HP-1 bonded phase fused silica capillary column (50 m × 0.32 mm i.d., film thickness 0.52 µm). The oven temperature was maintained at 30°C for 1 min, programmed at 5°C min/min to 150°C, and held for 0.1 min, then 10°C min/min, then 10°C min/min to 230°C. The carrier gas was hydrogen. Data were analyzed using HP Chemstation software. Quantification of VOCs was performed using a single point external standard quantification method using authentic samples of standards (Skelton et al., 2010).

Electrophysiology Lacewings, *Chrysoperla lucasina*, were obtained as adults from Koppert B.V., The Netherlands, and maintained at 20°C, 16 hr day length, prior to use. Electroantennogram (EAG) recordings were made using Ag-AgCl glass electrodes filled with a saline solution (composition as in Maddrell 1969, but without glucose:7.55 g/l sodium chloride, 0.64 g/l potassium chloride, 0.22 g/l calcium chloride, 1.73 g/l magnesium chloride, 0.86 g/l sodium bicarbonate, 0.61 g/l sodium orthophosphate). An antenna from a female lacewing was excised, its tip removed to ensure a good contact, and then suspended between the two electrodes. The signals were passed through a high impedance amplifier (UN-06, Syntech, Hilversum, The Netherlands) and analyzed with a customized software package (Syntech).

Coupled Gas Chromatography-Electroantennography (GC-EAG) The coupled GC-electrophysiology system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). The effluent from the transfer line to the antenna was delivered into a purified airstream (1 l/min) flowing continuously over the preparation. Separation of the VOCs was achieved on an AI 93 GC equipped with a cold on-column injector and an FID. The GC column was 50 m × 0.32 mm i.d. HP-1. The oven temperature was maintained at 30°C for 2 min and then programmed at 15°/min to 250°C. The carrier gas was helium. The outputs from the EAG amplifier and the FID were monitored simultaneously and analyzed using the Syntech software package. FID peaks were assumed to be EAG active if they elicited responses on three or more preparations.

GC-MS Analysis Coupled GC-MS analysis of volatile extracts was performed using a fused silica capillary column (50 m × 0.32 mm i.d., film thickness 0.52 µm, DB-1), attached to a cool on–column injector, which was directly coupled to a magnetic sector mass spectrometer (Autospec Ultima, Fisons Instruments, Manchester, UK). Ionization was by electron impact (70 eV, source temperature 250°C). Helium was the carrier gas. The oven temperature was maintained at 30°C for 5 min, and then programmed at 5°C/min to 250°C. Tentative identifications were made by comparison of spectra with mass spectral databases (NIST 2005). Identifications were confirmed by peak enhancement on GC using authentic compounds (Pickett, 1990).

Chemicals Porapak Q (80/100 mesh) was purchased from Alltech (PA, USA). Diethyl ether (purris.) was purchased from Sigma Aldrich. Hexane (95% pesticide residue) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Both solvents were distilled prior to use. (*Z*)-3-Hexenyl acetate and methyl salicylate were purchased from Sigma Aldrich (Steinheim, Germany). (*E*)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) (>98%) and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (>98%) were synthesized from geraniol and (*E,E*)-farnesol, respectively, by oxidation to the corresponding aldehyde followed by Wittig methylenation (Leopold, 1990).

Results

Olfactometry Experiments—Series 1 Alate (winged) *A. gossypii* spent more time ($P<0.001$) in the presence of odor from intact cotton seedlings compared to clean air (Fig. 1), and less time ($P<0.01$) in the presence of odor from *A. gossypii* infested cotton seedlings (Fig. 1) compared to clean air. *A. gossypii* also spent more time ($P<0.05$) in the presence of VOCs collected from intact cotton plants, but less time ($P<0.01$) in the presence of VOCs from *A. gossypii* infested seedlings, compared to solvent controls (Fig. 1).

VOC Analysis GC and coupled GC-MS analysis of headspace collections from 3-wk-old intact and *A. gossypii* infested cotton seedlings revealed an increase in the production of (*Z*)-3-hexenyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), methyl salicylate, and (*E,E*)-

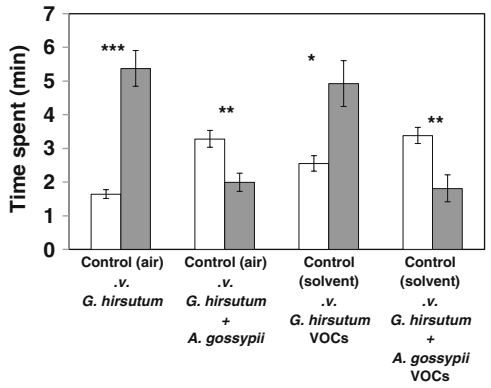


Fig. 1 Response in a four-arm olfactometer of the cotton aphid, *Aphis gossypii*, to odor from above *A. gossypii* infested or intact cotton (*Gossypium hirsutum*) plants, and VOCs obtained by air entrainment. Data are expressed as the mean (± SE) time (min) spent in treatment and control arms, and were analyzed by using a paired *t*-test (one tail) with statistical software (Genstat Version 11, VSN International Ltd.) after ensuring that data were normally distributed. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. □ Control. ■ Treatment

4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) in *A. gossypii* infested plants compared to uninfested plants (Fig. 2). Using a single point external standard quantification method with authentic samples of standards, the concentrations of *(Z)*-3-hexenyl acetate, DMNT, methyl salicylate, and TMTT in the *A. gossypii* infested sample

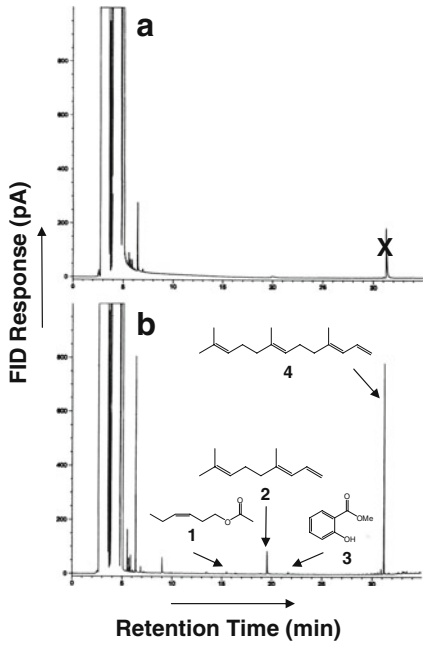


Fig. 2 Gas chromatography analysis of VOC samples collected from (**a**) uninfested and (**b**) *Aphis gossypii* infested cotton (*Gossypium hirsutum*) plants: *1* = (*Z*)-3-hexenyl acetate, *2* = (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), *3* = methyl salicylate, and *4* = (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). X = column bleed

were determined to be 0.34, 4.12, 0.33, and 14.62 ng/µl, respectively. Based on the weight of sampled plant material and the duration of entrainments, emission rates for the four compounds were calculated to be 3.54, 42.92, 3.44, and 152.29 ng/g/h, respectively.

Olfactometry Experiments—Series 2 A. gossypii spent more time ($P<0.05$) in the control arms when presented with a synthetic blend of (*Z*)-3-hexenyl acetate, DMNT, methyl salicylate, and TMTT, prepared and tested at the same dose and ratio as found in *A. gossypii* infested cotton seedlings (Fig. 3a). *A. gossypii* also spent more time ($P<0.05$) in the control arms when presented with the VOCs from intact cotton in the presence of the four component blend (Fig. 3a). When (*Z*)-3-hexenyl acetate, methyl salicylate, and TMTT

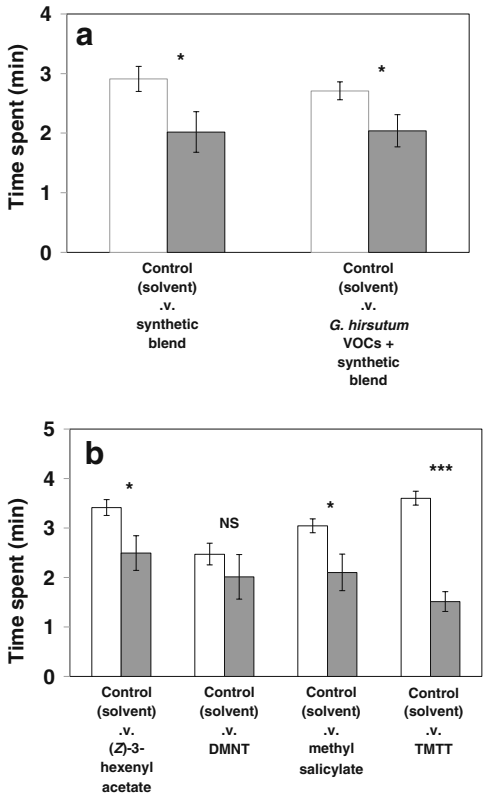


Fig. 3 Response in a four-arm olfactometer of the cotton aphid, *Aphis gossypii*, to (**a**) a synthetic blend of *Aphis gossypii*-induced compounds from cotton [(*Z*)-3-hexenyl acetate, DMNT, methyl salicylate, and TMTT] and a VOC sample collected from intact cotton in combination with the synthetic blend, and (**b**) to individual synthetic compounds. In all experiments, the synthetic compounds were tested at the levels found in *A. gossypii* infested cotton (0.34, 4.12, 0.33, and 14.62 ng μl^{-1} for (*Z*)-3-hexenyl acetate, DMNT, methyl salicylate, and TMTT, respectively). Data are expressed as the mean (± SE) time (min) spent in treatment and control arms, and were analyzed using a paired *t*-test (one tail) with statistical software (Genstat Version 11, VSN International Ltd.) after ensuring that data were normally distributed. * $P<0.05$; *** $P<0.001$. □ Control. ■ Treatment

were presented individually at doses found in the *A. gossypii* infested sample, *A. gossypii* spent more time in the control arms [Fig. 3b; (*Z*)-3-hexenyl acetate ($P<0.05$); methyl salicylate ($P<0.05$); TMTT ($P<0.001$)]. No significant difference was observed in the time spent between control and treatment arms when DMNT was tested.

Olfactometry Experiments—Series 3 When (*Z*)-3-hexenyl acetate, methyl salicylate, and TMTT were presented individually at a 1 μg dose, *A. gossypii* spent more time in the control arms [Fig. 4; $P<0.01$ for (*Z*)-3-hexenyl acetate; $P<0.001$ for methyl salicylate; $P<0.01$ for TMTT]. No significant difference was observed in the time spent when DMNT was tested. Similarly, addition of (*Z*)-3-hexenyl acetate, methyl salicylate, and TMTT individually to VOCs collected from uninfested cotton at a 1 μg dose resulted in *A. gossypii* spending more time in the control arms [Fig. 4b, $P<0.01$ for (*Z*)-3-hexenyl acetate; $P<0.001$ for methyl salicylate; and $P<0.05$ for TMTT]. There was no significant difference in the time spent by *A. gosspyii* between the control arms and the VOCs from uninfested cotton in the presence of DMNT.

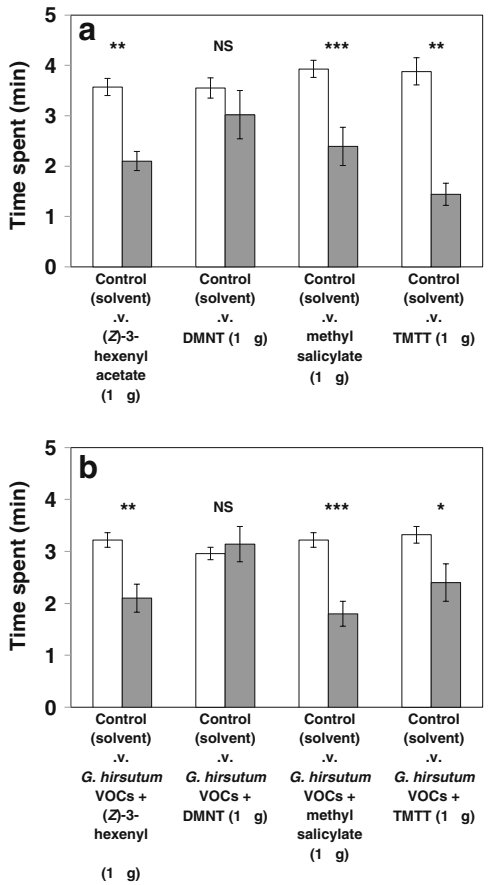


Fig. 4 Response in a four-arm olfactometer of the cotton aphid, *Aphis gossypii*, to (**a**) synthetic compounds tested alone (1 μg), and (**b**) a VOC sample collected from intact cotton (*Gossypium hirsutum*) plants in combination with individual synthetic compounds (1 μg). Data are expressed as the mean (± SE) time (min) spent in treatment and control arms, and were analyzed by using a paired *t*-test (one tail) with statistical software (Genstat Version 11, VSN International Ltd.) after ensuring that data were normally distributed. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; *NS* no significant difference. □ Control. ■ Treatment

Coupled GC-Electrophysiology Coupled GC-EAG analysis using the antennae of female *C. lucasina* revealed the perception of a number of compounds in the *A. gossypii* infested cotton VOC sample (Fig. 5). These included methyl salicylate, DMNT, and TMTT, but no significant response to (*Z*)-3-hexenyl acetate was observed in these experiments. Electrophysiological responses to several other peaks were demonstrated, with compounds tentatively identified as 6-methyl-5-hepten-2-one, (*Z*)-4,8-dimethyl-1,3,7-nonatriene, octanal, nonanal, decanal, 3-methylhexane, *n*-heptane, methylcyclohexane, α-pinene, and an ethylbenzaldehyde isomer (stereochemistry undefined).

Discussion

To our knowledge, this is the first study of the chemical ecology underlying interactions between cotton and the cotton aphid, *A. gossypii*, and the induction of defense VOCs following *A. gossypii* infestation. Olfactometer data confirmed that *A. gossypii* was attracted to the odors from uninfested cotton plants but was repelled by odors from *A. gossypii* infested plants. Furthermore, *A. gossypii* was attracted to VOCs collected from uninfested cotton but repelled by those from *A. gossypii* infested cotton, and also was repelled by three synthetic VOCs identified as being induced by *A. gossypii* infestation, i.e., (*Z*)-3-hexenyl acetate, methyl salicylate, and TMTT. No effect was observed for DMNT, i.e., the lower molecular weight homologue of TMTT, which was also induced on aphid infestation. Induction of defense compounds in cotton on herbivory by beet armyworms (*Spodoptera* spp.) and whiteflies has been reported previously (Loughrin et al., 1994; McCall et al., 1994; Rose et al., 1996; Rodriguez-Saona et al., 2003; Rose and Tumlinson, 2005), and include three of the defense VOCs reported in this study, i.e., the green leaf volatile compound (*Z*)-3-hexenyl acetate, and the homoisoprenoid compounds DMNT and TMTT. In our study, methyl salicylate was emitted by *A. gossypii* infested cotton, but no emission of sesquiterpenes was detected. This emission pattern by *A. gossypii* infested cotton implies an herbivore specific response, i.e., a response to an aphid (sucking) pest. The emission of methyl salicylate in this study is similar to that seen for aphid damaged soybean (Zhu and Park, 2005).

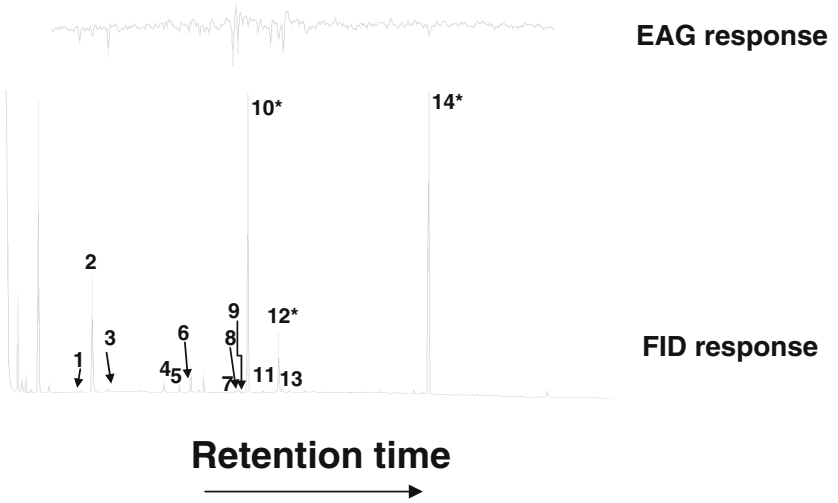


Fig. 5 Typical coupled GC-EAG analysis showing antennal response of female lacewings, *Chrysoperla lucasina,* to the headspace sample from *Aphis gossypii* infested cotton (*Gossypium hirsutum*) plants. Upper trace, response of antenna; lower trace, FID response. GC peaks identified as: *1* = 3-methylhexane; *2* = *n*-heptane; *3* = methylcyclohexane; *4* = α-pinene; *5* = 6-methyl-5-hepten-2-one; *6* = octanal; *7* = linalool oxide (furan); *8* = nonanal; *9* = (*Z*)-4,8-dimethyl-1,3,7-nonatriene; *10* = (*E*)-4,8-dimethyl-1,3,7-nonatriene; *11* = 2, 3 or 4-ethylbenzaldehyde; *12* = methyl salicylate; *13* = decanal; *14* = (*E, E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. *Identifications confirmed by GC peak enhancement using authentic standards

Blends and ratios of VOCs are important in insect olfaction (Bruce et al., 2005), and the VOC blend released by *A. gossypii* is likely to be important for modifying aphid natural enemy behavior, as seen for other aphid/plant interactions (Du et al., 1998). The profile of induced VOCs also may vary, based on age/stage of the plant (Zhu and Park, 2005), along with the type of cultivar studied. Thus, selection of cultivar specific traits such as the production of induced defense VOCs is an important point to be considered for future cultivar development in relation to biological control. Furthermore, studies have shown that induced defense VOCs have the ability to prime neighboring plants for enhanced defense, including one of the induced VOCs in this study, i.e., (*Z*)-3-hexenyl acetate (Frost et al., 2008). Further studies are planned to investigate the potential for induced cotton VOCs to prime neighboring plants for defense against future biotic (herbivore or pathogen) attack.

VOC production in crop plants, e.g., maize, is induced by elicitors originating from herbivores (Alborn et al., 1997; Frey et al., 2000). Furthermore, VOC production is linked to transcription of terpene synthase genes, which are involved in the biosynthesis of sesquiterpenes, and of which several have been isolated and characterized (Degenhardt et al., 2009). Production of the homosesquiterpene compound TMTT in *Arabidopsis thaliana* involves *TPS04/GES*, a geranyllinalool synthase (Herde et al., 2008), and CYP82G1, a cytochrome P450 monoxygenase, the latter reported as being responsible for the oxidative cleavage of geranyllinalool (Lee et al., 2010). However, other genes may be involved, including other cytochromes P450, e.g., CYP81D11 (Matthes et al., 2011). Production of methyl salicylate is under the control of salicylate methyltransferase (SMAT) in tobacco (Ross et al., 1999) and the *bsmt1* gene in *A. thaliana* (Snoeren et al., 2010). Further studies at the molecular level are required to establish the molecular basis of VOC production in cotton.

The EAG active compounds (*Z*)-3-hexenyl acetate, methyl salicylate, and TMTT all interfered with the attraction of *A. gossypii* to VOCs from uninfested cotton. Roles for these three compounds in the chemical ecology of plant/aphid/natural enemy interactions have been reported previously. Hardie et al. (1994) demonstrated that methyl salicylate stimulates olfactory cells in the primary rhinaria of the black bean aphid, *A. fabae.* In behavioral studies employing a linear track olfactometer, this compound was repellent to *A. fabae* and also inhibited attraction to VOCs from the host plant, broad bean, *Vicia faba.* Methyl salicylate also is released from soybean, *Glycine max*, following herbivory by the soybean aphid, *A. glycines*, and is shown to be electrophysiologically active for seven-spot ladybirds, *Coccinella septempunctata* (Zhu and Park, 2005). Thus, release of methyl salicylate by cotton on damage by aphid pests may be a well-defined strategy that attracts predators. Application of methyl salicylate in wheat fields reduced aphid populations by at least by 30–40% when used as a slow release formulation (Pettersson et al., 1994). Increased biological control activity with methyl salicylate has been reported in hop yards and grape blocks (James et al., 2005, Zhu and Park, 2005). *Aphis fabae* is repelled by (*Z*)-3-hexenyl acetate and TMTT (Webster et al., 2010), in line with the present findings, and the latter is

attractive for aphid parasitoids (Bruce et al., 2008) and stink bug parasitoids (Moraes et al., 2009) following its emission from plants exposed to the plant activator *cis*-jasmone. Although the presence of specific olfactory receptors for TMTT has been demonstrated for an aphid parasitoid, *Aphidius ervi* (Bruce et al., 2008), to our knowledge, this paper is the first to report evidence of antennal perception of this plant stress compound in an aphid predatory species.

Coupled GC-electrophysiology with *C. lucasina* revealed the presence of other electrophysiologically active compounds for which no discernable difference in levels between uninfested and *A. gossypii* infested samples could be detected by GC analysis (data not shown). These included 6-methyl-5-hepten-2-one, which has been shown in other studies to be an important host derived semiochemical for *A. gossypii* (da Costa et al., 2011), while octanal and decanal have been shown to be host derived semiochemicals for *A. fabae* (Webster et al., 2010). Investigations into the behavioral roles of the EAG active compounds other than those used in behavioral studies, i.e., 6-methyl-5-hepten-2-one, (*Z*)-4,8-dimethyl-1,3,7-nonatriene, octanal, nonanal, decanal, 3-methylhexane, *n*-heptane, methylcyclohexane, α-pinene, and an ethylbenzaldehyde isomer (Fig. 5), were beyond the scope of this study, but will form part of ongoing investigations, both in the laboratory and in the field.

In summary, this study has demonstrated the role and identity of volatile semiochemicals released by cotton upon infestation by the aphid *A. gossypii* and olfactory detection of the defense compounds by predatory *Chrysoperla* spp. lacewings. While the manipulation of beneficial insects by using field deployment of volatile semiochemicals has been investigated extensively in various crop scenarios, the application of synthetic blends can be costly and inefficient due to the high cost of semiochemical production, coupled with the instability and high volatility of semiochemicals under field conditions. Production of defense semiochemicals in cotton for protection against *A. gossypii* could be achieved in a cheaper and more sustainable manner by using "smart" resistant cotton varieties, through activation and priming of defense by small lipophilic molecule plant activators such as *cis*-jasmone (Birkett et al., 2000; Bruce et al., 2003; Moraes et al., 2009), or even by developing cotton varieties that have been genetically transformed to produce these defense semiochemicals upon exposure to *cis*-jasmone (Bruce et al., 2008; Matthes et al., 2010). Further studies are underway to investigate the potential for activation of defense in cotton.

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Production of Induced Volatiles by *Datura wrightii* in Response to Damage by Insects: Effect of Herbivore Species and Time

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Abstract Natural enemies of herbivorous insects utilize numerous chemical cues to locate and identify their prey. Among these, volatile plant compounds produced after attack by herbivores may play a significant role (hereafter herbivore-induced plant volatiles or HIPVs). One unresolved question is whether the composition of the volatile cue blends induced by different herbivore species differ consistently enough to indicate not only that the plants are damaged by herbivores but also the identity of the herbivore species causing the damage. We studied HIPV production in the undomesticated plant species *Datura wrightii* in the laboratory when damaged by either of two leaf-chewing herbivore species, *Lema daturaphila* or *Manduca sexta*, or when damaged by *L. daturaphila* and the piercing-sucking bug, *Tupiocoris notatus*, or both *L. daturaphila* and *T. notatus,* for 24 hr. HIPV production was monitored 1 d before induction, the day of induction, and for 7 d after induction. In all experiments, both the quantities and composition of the HIPV blends varied with the time since induction as different components reached peak production at different times after induction. HIPV blends did not differ consistently with the herbivore species causing the damage. For plants damaged by both *L. daturaphila* and *T. notatus*, greater amounts of HIPVs were produced than by plants damaged by either species alone, but the amounts did not differ from that predicted as the sum from damage inflicted by each herbivore species independently. The HIPVs of *D. wrightii* are a general rather than specific indicator of damage by herbivores. Because generalist predators are the most abundant natural enemies in this system, general cues of herbivore damage may be all that are required to facilitate the discovery by predators of plants damaged by any of several suitable prey species.

Key Words Induced defense · HIPVs · Tritrophic interactions · Specificity · *Datura wrightii* · *Lema daturaphila* · *Tupiocoris notatus* · *Manduca sexta*

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Introduction

Odors of plants can serve as cues to attract natural enemies of the insect herbivores attacking those plants (Vinson, 1976, 1981; Nordlund et al., 1988; Allison and Hare, 2009). These odors may be comprised of hundreds of compounds (Dudareva et al., 2006), many of which are released only by plants after they have been damaged by herbivores (Elzen et al., 1983; Dicke and Sabelis, 1988; Turlings et al., 1991; Tumlinson et al., 1992). One unresolved question in the production of these herbivore-induced plant volatiles (HIPVs) is whether different herbivore species induce consistently different or specific blends, and how specific a blend must be in order to be recognized by the species of natural enemies that effectively suppress the plant's herbivores.

Congenitally fixed responses to herbivore-specific cues were predicted for cases involving extreme specialization by natural enemies on particular herbivore species which themselves are specialized on a particular species of plant, whereas progressively greater levels of learned responses

on the part of the natural enemies were predicted for less extreme levels of specialization (Vet and Dicke, 1992; Faeth, 1994). Similarly, specialist natural enemies were predicted to utilize specific cues from their host/prey complex, whereas generalists were predicted to utilize general cues released by all of their different host/prey complexes (Steidle and van Loon, 2003). Although simple predictions may be reasonable for relatively simple trophic chains, especially for plants attacked by a single herbivore species that itself has only one resident species of natural enemy, predictions that concern the responses of plants become more difficult if plants are attacked by a community of generalist and specialist herbivores, themselves attacked by numerous species of natural enemies differing in host specificity.

In some cases, attack by single vs. multiple herbivore species may result in statistically significantly different HIPV blends (Shiojiri et al., 2001; Delphia et al., 2007; De Boer et al., 2008), whereas in other systems, plants infested with multiple herbivore species simply emit a greater quantity of the same volatiles than plants damaged by single herbivore species (Moayeri et al., 2007). In still other systems, damage by one herbivore species may suppress the HIPV response normally induced by other species (Zhang et al., 2009). Therefore, the consequences of infestation of plants by more than one species of herbivore are difficult to predict, and depend upon such factors as the particular combination of plant species, herbivore species, and species of natural enemy (Hare, 2011).

Another controversy in this area focuses upon whether the HIPV blend produced by plants in response to damage by herbivores is anything more than a simple response to mechanical injury. In most studies, damage by herbivores elicits HIPV blends containing a greater range of compounds and in greater concentrations than mechanical damage. Mechanical damage that is most similar to damage by herbivores, both as to the mechanism of damage as well as its duration, elicits HIPV blends most similar to those elicited by natural herbivores (Mithofer et al., 2005; Connor et al., 2007). Plant species may differ in how closely their volatile profiles induced by mechanical damage resemble those induced by herbivore damage, however (Maffei et al., 2007). Even for plant species in which the volatile blends are similar in response either to mechanical or herbivore damage, differences in plant responses can be seen at the molecular level (Bricchi et al., 2010).

Variation in the total quantity of HIPVs as well as the relative proportions of components may vary with plant genotype (Hare, 2007; Schuman et al., 2009), the environmental conditions under which plants are grown (Gouinguene and Turlings, 2002; Kigathi et al., 2009), and the amount of time following damage (Turlings et al., 1998; Kunert et al., 2002). Variation in composition also may be a function of the amount of damage (Gouinguene et al., 2003; Delphia et al., 2007). Such variation raises important questions as to whether any aspect of the HIPV blends of plants induced by different herbivore species may be sufficient to reveal reliably the identities of the herbivores (Turlings et al., 1995; Turlings and Wäckers, 2004; Webster et al., 2010).

When damaged by its primary herbivore, the chrysomelid beetle *Lema daturaphila*, plants of the undomesticated plant species, *Datura wrightii* produce an array of at least 17 compounds, most of which are sesquiterpenes (Hare, 2007, 2010). In a laboratory study, total production of volatiles increased 3.9 to 16.2 times among genetic lines of *D. wrightii* after insect damage. The most abundant compound was (*E*)-β-caryophyllene, which comprised from 17 to 59% of the volatiles from insect-damaged plants, depending upon genetic line. Other relatively abundant HIPV compounds included (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, (*E,E*)-4,8,12-trimethyl-1,3,7,11- tridecatetraene (hereafter TMTT), (*E*)-4,8-dimethyl-1,3,7- nonatriene (hereafter DMNT), and β-selinene, most of which are commonly induced by herbivores in other plant species (Arimura et al., 2005). The array of compounds emitted by young plants induced in the field was similar (Hare, 2010; Hare and Sun, 2011).

Within southern California populations, *D. wrightii* exhibits two leaf trichome phenotypes, and aspects of the ecology and genetics of the trichome dimorphism have been described elsewhere (van Dam and Hare, 1998; van Dam et al., 1999). The 'velvety' phenotype is densely covered by short, non-glandular trichomes and feels velvety to the touch, whereas the 'sticky' phenotype is less densely covered with glandular trichomes that secrete esters of glucose and aliphatic acids, and these leaves feel sticky to the touch. The trichome phenotype is governed by a single locus and is inherited in a Mendelian fashion; the allele for the sticky phenotype is dominant over that of the velvety phenotype (van Dam et al., 1999).

The herbivore community that attacks *D. wrightii* in southern California is relatively simple, being comprised of some 5–10 species depending upon season and location, but the herbivore community varies with trichome phenotype (Elle and Hare, 2000; Hare and Elle, 2002). The most abundant herbivore species overall is *L. daturaphila*, a folivore whose larval and adult stages damage sticky and velvety plants similarly throughout the growing season. Another potentially more damaging insect herbivore, because of its greater rate of food consumption (Bronstein et al., 2006), is the tobacco hornworm, *Manduca sexta*. This species is more constrained seasonally in its abundance on *D. wrightii,* but it is more abundant on velvety than sticky plants (Elle and Hare, 2000; Hare and Elle, 2002).

The most abundant herbivore on sticky plants is the mirid bug, *Tupiocoris notatus*. It utilizes the sugar esters produced by the glandular trichomes of the sticky phenotype of *D. wrightii* as feeding and oviposition stimulants, but *T. notatus* can survive and reproduce on velvety plants if given no choice (van Dam and Hare, 1998). Although the effect of *T. notatus* on HIPV production of *D. wrightii* has not yet been studied, the omnivorous heteropteran predator, *Geocoris pallens*, is attracted to plants of *Nicotiana attenuata* damaged either by *T. notatus* or *Manduca* spp. (Kessler and Baldwin, 2004). On the other hand, damage by *T. notatus* also can suppress some of the jasmonate-mediated responses to other insects in other plant species (Heidel and Baldwin, 2004), which suggests that the HIPV blend induced by *T. notatus* in combination with *L. daturaphila* may differ from the HIPV blend induced by *L. daturaphila* alone.

A number of natural enemies that differ in host range are associated with these herbivore species. In a comprehensive survey of the parasitoids and predators of *M. sexta* on *D. wrightii* in Arizona, more than 40 natural enemies were identified, including 34 insect predators and parasitoids from 8 different orders and 14 species of spiders from 4 different families (Mira and Bernays, 2002). Less intensive sampling of the natural enemies associated with *D. wrightii* in California found many of the same generalist predators, including species of *Nabis* and *Chrysoperla* (Gassmann and Hare, 2005), although the most abundant predator in California was *G. pallens*, a species not reported from the survey in Arizona. Like several other generalist predators, *G. pallens* exhibited lower rates of predation of *L. daturaphila* and *T. notatus* when the herbivores were on sticky plants than when on velvety plants in the greenhouse (Gassmann and Hare, 2005). This predator also inflicted greater levels of mortality of young larvae of *L. daturaphila* on young *D. wrightii* plants when plants either were damaged by *L. daturaphila* or treated with MeJA compared to undamaged or untreated plants. The increase in predation was especially pronounced on the velvety genotype (Hare and Sun, 2011). In field experiments, predation of eggs of *L. daturaphila* by *G. pallens* increased with increasing total HIPV production in the early part of the season, when predators were colonizing plants, but predation rates did not vary with the variation in the composition of the HIPV blend produced by different genetic lines of *D. wrightii* (Hare and Sun, 2011). Common parasitoids of *M. sexta* in California include the generalists, *Hyposoter exiguae* and *Trichogramma* spp. (Oatman et al., 1983). Only two parasitoids of *L. daturaphila* are observed occasionally in southern California, *Conura delumbis* (Hymenoptera: Chalcidae) and *Myiopharus infernalis* (Diptera: Tachinidae). Both of these utilize other coleopteran hosts as well (Arnaud, 1978; Burks, 1979).

Our overall goals were to compare the quantities and compositions of HIPV blends induced by the three predominant herbivores of *D. wrightii* in southern California, the chewing herbivores *L. daturaphila* and *M. sexta*, and the piercing-sucking herbivore, *T. notatus,* when these insects were feeding on two relatively highly inducible lines of *D. wrightii*. We asked if the blends differed consistently among inducers to indicate the possible identity of the herbivore causing the damage. The paucity of specialized natural enemies of the herbivores of *D. wrightii* compared to the abundance of generalist predators also suggested that there may be reduced opportunities for the evolution of herbivore-specific HIPV blends in *D. wrightii.* We also asked if the quantities and composition of HIPV blends induced by plants infested by both *T. notatus* and *L. daturaphila* differed from the blends induced by each species alone. Both the total quantity as well as the composition of blends induced by each herbivore treatment were subjected to multivariate analysis and compared over a period from 1 d prior to induction until 7 d after induction.

Methods and Materials

Two experiments were carried out using similar, though not identical designs. The first compared the HIPVs induced over time by *L. daturaphila* and *M. sexta,* whereas the second compared the HIPVs induced by *T. notatus* and *L. daturaphila* when each damaged plants alone or in combination.

Plants Fifth-generation backcross progeny of the highly inducible MVV6 and MVV8 lines (Hare, 2007) were used in both experiments. These lines were established by backcrossing heterozygous sticky progeny to their original velvety pollen parent. Because the genotypes of the progeny of these backcrosses can be only heterozygous or homozygous recessive, then trichome phenotype maps directly to genotype. The expected ratio of adult heterozygous sticky to homozygous recessive velvety sibs within each of the lines is 1:1, and sibs within families are expected to be at least 98.4% similar after five generations of backcrossing. Plants were grown in a greenhouse equipped with high-pressure sodium lamps providing supplemental illumination for 14 hr/d so that mid day light intensities averaged 1250±39 μmol m^{-2} s^{-1} PAR illumination (N=5 readings) at plant height. Greenhouse temperatures ranged between 15° and 35°C. Plants were maintained under these conditions for ca. 2–4 mo until they had produced 6–8 true leaves (first experiment) or 10–12 leaves (second experiment). The first experiment was performed using younger trichome-undifferentiated plants

because prior research showed no difference in HIPV blends between sticky and velvety plants when induced by *L. daturaphila* (Hare, 2007). The effect of trichome genotype was of more interest with regard to the HIPVs induced by *T. notatus*, so the second experiment utilized older plants for which trichome differentiation had been completed (van Dam et al., 1999).

Collection of Volatiles Volatiles were collected nondestructively from intact plants in a growth room 14:10 photophase: scotophase, 26°C day and 20°C night temperatures with light intensity of 293 (± 29 SE, n=5) μmol m^{-2} s^{-1} PAR illumination at plant height. Aeration chambers were made from polyester cooking bags (unprinted 45×55 cm bags, Terinex, Bedford, England) as described earlier (Hare, 2007).

An aeration chamber was placed over a whole plant, excluding senescing leaves, and secured with a twist-tie around the stem. Intake and exhaust flow rates were regulated with flow meters (Aalborg, Orangeburg, NY, USA). PTFE tubing was used upstream of the adsorbent trap and PVC tubing was used downstream. Compressed air, filtered through activated charcoal, flowed into the bag at 600 ml/min. Periodic collections of volatiles from an empty bag showed that the charcoal filter removed all potentially interfering volatile compounds. Air was exhausted from the bag through an adsorbent trap at the same rate using house vacuum. HIPVs were collected on traps made of glass tubing and filled with Super-Q (25 mg, Alltech, State College, PA, USA). At the termination of the aeration, traps were wrapped in aluminum foil, labeled, and stored at −20°C for extraction later that day.

For the first experiment, 6 plants were assigned to each of two herbivore damage treatments, and 1 plant was assigned to a 'no induction' control treatment within each trial. Control plants were included simply to ensure that plants were not induced by mechanical handling. The greater allocation of replicates to insect-damage treatments reflect the higher priority placed upon comparing the HIPV blends induced by the two different herbivore species than on comparing either to uninduced controls. The whole experiment was repeated five times. One plant in one trial was damaged during the course of the experiment and deleted, leaving a total of 64 experimental plants. For the second experiment, 4 plants, 2 sticky and 2 velvety were assigned to damage by *T. notatus* alone, *L. daturaphila* alone, or both, whereas 1 plant of each trichome genotype was assigned to a 'no induction' treatment (control) within each trial of 14 plants. This experiment also was repeated five times, but 3 plants were damaged during handling and so were deleted from the experiment leaving a total of 67 plants.

Induction Treatments For plants to be induced by *L. daturaphila,* groups of four fourth (last) instar larvae of *L. daturaphila* per leaf were placed on each of three leaves, and larvae were allowed to feed for 24 hr. The youngest full-sized leaf, plus the one above and the one below, were chosen, and larvae were confined to their leaf by a small organdy leaf cage. For the plants to be induced by *M. sexta*, one newly-molted third instar *M. sexta* per leaf was placed on each of three leaves of similar age, as above, and these larvae also were confined to their leaf with a small leaf cage. These numbers and developmental stages of the two insect species were chosen on the basis of preliminary experiments (not shown) to cause similar levels of damage. In the second experiment, for plants to be induced by *T. notatus,* plants were infested with 50 nymphs and adults of *T. notatus*, two pairs of adult *L. daturaphila*, or both 50 *T. notatus* individuals and two pairs of adult *L. daturaphila*, and these insects also were allowed to feed on plants for 24 hr. A single 24-hr-damage-period was chosen to facilitate the calculations of the rate of HIPV emission per unit of remaining plant tissue over time. Although it might have been more realistic to leave insects on the plants until they had ceased feeding, this would have caused an artificial reduction in HIPV production by these plants over time due to the smaller quantities of leaf tissue available to emit HIPVs on each day of the 8-d-observation period.

Preliminary experiments showed little difference in HIPV quantities induced by 25, 50, or 100 *T. notatus* per plant, and the middle density of *L. daturaphila* was chosen to minimize leaf tissue losses during the experiment. The treatment with both herbivore species was designed to compare directly the quality and composition of blends induced by both herbivore species with the HIPV blends elicited by the same numbers of each herbivore species alone. In prior experiments, both adults and larvae of *L. daturaphila* induced the production of similar groups of HIPVs. In total, plants were aerated continuously for 9 d, including the day before induction, the day of induction, and then daily aerations for 7 d following induction, and traps were changed every 24 hr.

At the end of the experiment, all aerated leaves were removed from the plant, and the leaves damaged by *L. daturaphila* or *M. sexta* were scanned and digitized to determine the amount of leaf area removed using the Scion Imaging Software for Windows ver. A.4.0.3.2, (Scion Corp., Frederick, MD, USA). Because of the piercing-sucking feeding mode of *T. notatus,* no attempt was made to quantify damage caused by this insect. After scanning, all aerated leaves were dried (80°C for 48 hr) and weighed to determine total aerated leaf dry mass. Plant growth was slight over the course of the experiment and was ignored in determining total aerated leaf dry mass.

Volatiles Analysis Volatiles were eluted from the traps with 150 μl of CH_2Cl_2 containing 4 ng/μl of 1-bromoheptane

(Sigma-Aldrich) as an internal standard into autosampler vials with 250 μl glass inserts, and vials were sealed with crimp caps and PTFE-lined rubber septa. Samples were analyzed by gas-liquid chromatography largely as described in Hare (2007) except that a shorter column (DB-5, 30 m, 0.25 mm ID, 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA) and an autoinjector (Hewlett-Packard 7673) were used. To confirm identifications, selected samples were analyzed with a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 mass selective detector, and the mass spectra and retention times of compounds were matched with those of authentic standards as described in Hare (2007). Peaks were quantified in units of ng g^{-1} leaf (dry wt.) hr^{-1} using Agilent ChemStation® software based on comparison of the peak height of each HIPV component with that of the internal standard.

Statistical Analyses We performed an exploratory principal components analysis (PCA) using PROC PRINCOMP of SAS (SAS Institute, 2008). The covariance matrix of $\log_{10}$ –transformed quantities of the individual HIPVS from induced plants was used to calculate a smaller number of uncorrelated components that were linear, independent combinations of the original variables. The factor loadings show how much each of the original variables contributes to each new component, and factor scores for the new components can be calculated for each original observation. This allows one to examine the composition of the blend as a whole instead of as an *ad hoc* combination of individual, potentially correlated variables. A repeated measures analysis of covariance on these factor scores (PCA-ANCOVA) was used in the experiment comparing the HIPV blends induced by *M.* sexta with *L. daturaphila* to detect significant variation in the composition of the blends induced by the different herbivore species. The analysis was performed using PROC MIXED of SAS that included: 1) insect species, aeration day, and their interaction as fixed effects; 2) block as a random effect; 3) percent leaf area removed as a covariate; and 4) the individual plant as the repeated subject effect over the 9 d of aeration. The results from PCA then guided analyses of individual compounds to explore changes in the composition of the HIPV blend with herbivore species or over time. Where significant interactions were obtained, then they were decomposed by using the SLICE option of PROC MIXED. Similar repeated-measures analyses were performed on the data from the experiment by comparing HIPV blends from plants damaged by *L. daturaphila, T. notatus,* or both except 1) there was no need to include defoliation level as a covariate, and 2) the effect of trichome genotype and all interactions with trichome genotype were included as fixed effects.

To test for nonadditive effects on the total HIPV production of both *L. daturaphila* and *T. notatus* feeding simultaneously on *D. wrightii*, we calculated the expected HIPV production of plants damaged by each herbivore species alone for each sampling day and block. These expected values were calculated after pooling plants from both trichome genotypes because HIPV production did not differ between trichome genotypes (see Results). The observed HIPV production of plants damaged by both herbivore species simultaneously was compared to these expected values, and the deviation from the expected value was calculated by subtraction. The deviations were not amenable to parametric statistical analysis because they did not meet the assumption of normally-distributed errors and homogeneity of variances, and no common transformation rectified these problems. We, therefore, utilized a non-parametric method of analysis. The frequencies of plants producing greater or lesser quantities of HIPVs than predicted was analyzed by a replicated goodness-of fit *G*-test (Sokal and Rohlf, 1995) replicated over each sampling day. Under the null hypothesis of additive effects of the two herbivore species on HIPV production, the frequencies of plants producing greater quantities of HIPVs than predicted would not differ significantly from the frequencies of plants producing less HIPVs than predicted; synergism would be consistent with a significantly greater number of plants producing higher quantities of HIPVs than predicted, and antagonism would be consistent with a significantly greater number of plants producing lower quantities of HIPVs than predicted. Data for the pretreatment aeration were not included in this analysis.

Results

Lema and Manduca A preliminary analysis of total HIPV production showed that HIPV production was low and did not differ among plants assigned to the various experimental treatments on the day prior to induction ($F_{2, 57}$=0.74, P=0.48, Fig. 1a). HIPV production of control plants did not differ among days ($F_{8, 535}$=0.81, P=0.59), whereas that of the herbivore-damaged plants did differ over time ($F_{8, 535}$=13.49, P<0.001 for plants induced by *L. daturaphila* and $F_{8, 535}$= 12.38, P<0.001 for plants induced by *M. sexta*). Highest average total HIPV production of 279±70 ng HIPVs g^{-1} hr^{-1} occurred in the plants damaged by *L. daturaphila* 1 d after induction (Fig. 1a). Because the control plants remained uninduced through the experiment, they were deleted to simplify subsequent analyses of potential differences in the HIPV quantities and blends induced by the two different herbivore species. Mean leaf area removed by *L. daturaphila* was 10.1±1.0 cm^2, or 20.0±2.5% of the available leaf area.

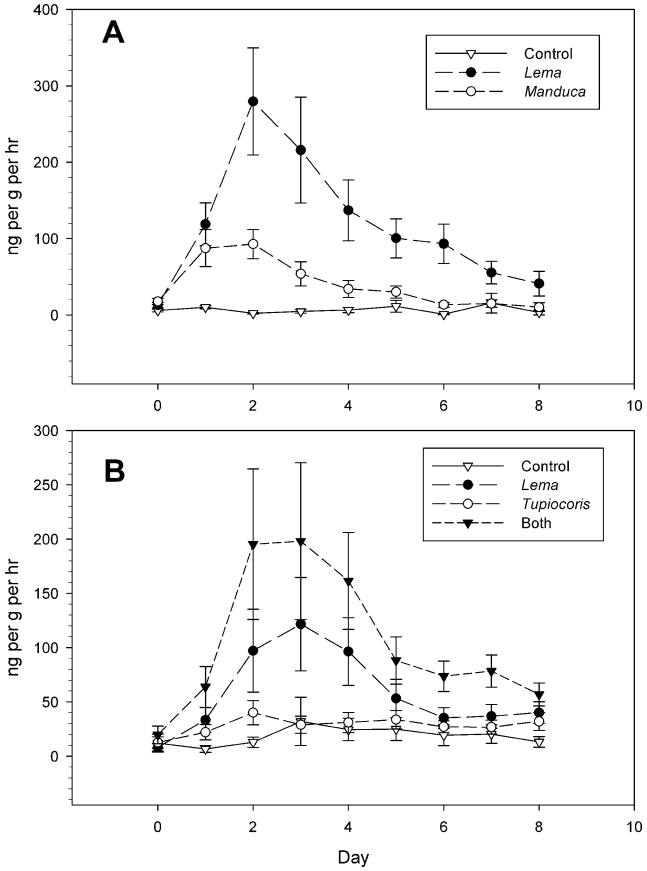


Fig. 1 Mean (± s.e.) total HIPVs by day and herbivory treatment for plants damaged for 24 hr between Day 0 and Day 1. Plot **a**: plants damaged by 12 fourth-instar *Lema daturaphila* or three third-instar *Manduca sexta* larvae; plot **b**: plants damaged by four *Lema daturaphila* adults, 50 adults and nymphs of *Tupiocoris notatus*, or the same numbers of both insect species simultaneously

Mean leaf area removed by *M. sexta* was less than expected based upon the preliminary experiment (5.9± 1.0 cm^2 or 10.1±1.3% of available leaf area), but the distributions of leaf area removed by the two insect species overlapped, with a maximum leaf area removed of 46% for *L. daturaphila* and 31.5% for *M. sexta*. In general, the group of HIPVs obtained was similar to those reported in a previous laboratory study (Hare, 2007). Abundant HIPVs included (*E*)-β-ocimene, (*E*)-β-caryophyllene, (*E,E*)-α-farnesene, (*Z*)-3-hexenyl acetate, and DMNT. Other components included limonene, linalool, an isomer of bergamotene, α-humulene, β-selinene, nerolidol, TMTT, and methyl salicylate (MeSA).

Results of the PCA analysis on HIPVs showed that the first component (PC 1) accounted for 63% of the total variation and PC 2 accounted for an additional 12%. PC 3 accounted for 5.5% and the remaining PCs accounted for progressively smaller percentages of the total variance and will not be considered further. All original variables had positive loadings on PC1, thus indicating that this component is largely a "general production" factor much like a "general size" factor in morphological studies (Fig. 2a). The highest loadings were for DMNT, TMTT, (*E,E*)-α-farnesene, (*E*)-β-ocimene, (*Z*)-3-hexenyl acetate, and (*E*)-β-caryophyllene (Fig. 2a). PC 2 is an index of the composition of the blend and contrasted the abundance of TMTT with DMNT, and to a lesser extent, TMTT with (*E,E*)-α-farnesene, (*E*)-β-ocimene, (*Z*)-3-hexenyl acetate, and (*E*)-β-caryophyllene. Most of the rest of the components had factor loadings near zero on PC 2 (Fig. 2b). PC 3 independently contrasts plants producing relatively high levels of (*E*)-β-caryophyllene with plants producing relatively high levels of MeSA, (*Z*)-3-hexenyl acetate, and DMNT (Fig. 2b).

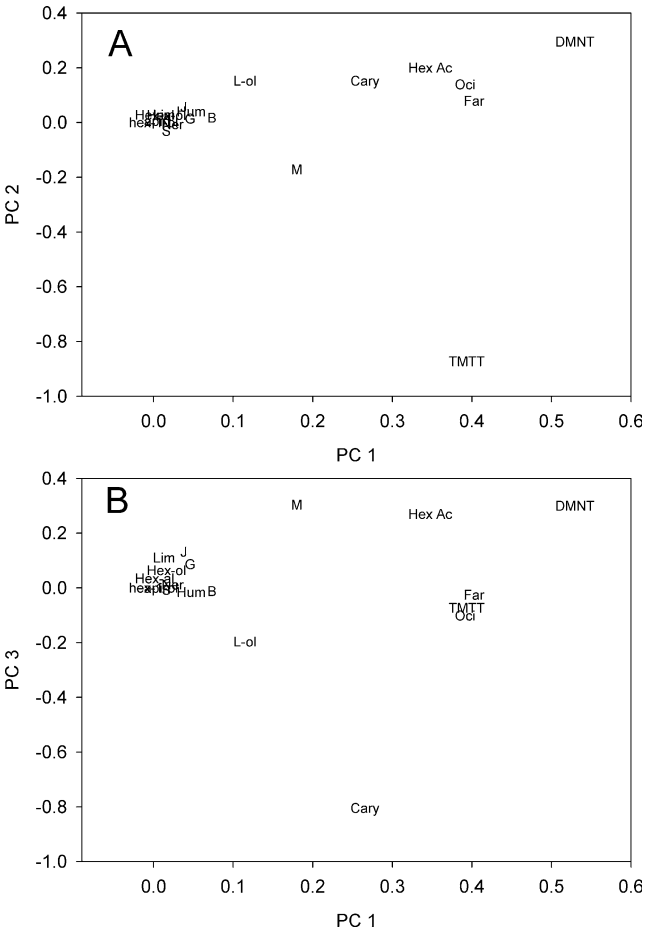


Fig. 2 Factor loadings for individual compounds for plants damaged either by larvae of *Lema daturaphila* or *Manduca sexta* for plants damaged for 24 hr between Day 0 and Day 1 as in Fig. 1a. Plot **a**: PC1 and PC 2; plot **b**: PC1 and PC 3. Chemical abbreviations: Cary: (*E*)-β-caryophyllene; DMNT: (*E*)-4,8-dimethyl-1,3,7-nonatriene; Far: (*E,E*)-α-farnesene; Hex Ac: (*Z*)-3-hexenyl acetate; Oci: (*E*)-β-ocimene; Lol: linalool; M: methyl salicylate; TMTT: (*E*)-4-8,12-trimethyl-1,3,7,11-tridecatetraene. Overlapping near the origin: Hum: α-humulene; J: jasmone; Lim: limonene; Ner: nerolidol Hex-al: *trans*-2 hexenal; Hex-ol: *cis*-3-hexen-1-ol; Hex-1-ol: *trans* 2-hexen-1-ol; apin: α-pinene B: α-bergamotene; G: geranyl acetone S: β-selinene

Scores for PC 1 increased with increasing defoliation (test of covariate, $F_{1,\ 506}$=7.87, P=0.005). There were no differences overall in the factor scores for PC 1 between the two induction treatments ($F_{\ 1,\ 506}$=0.00, P=0.97) after correcting for differences in defoliation, but there was significant variation in factor scores over time ($F_{8,\ 506}$=19.14). Scores for PC 1 also differed significantly due to the day by treatment interaction ($F_{\ 8,\ 506}$=3.82, P=0.002, Fig. 3a). Decomposing this interaction showed that PC 1 scores differed between induction treatments starting on the day after damage and continued to differ 6 d after insects were removed (all $P\leq$0.005). During this time period, plants induced by *M. sexta* had significantly lower scores for PC 1 than plants induced by *L. daturaphila*, even after correcting for the differences in defoliation; per unit leaf area removed, *M. sexta* induced a lower production of HIPVs than did *L. daturaphila*.

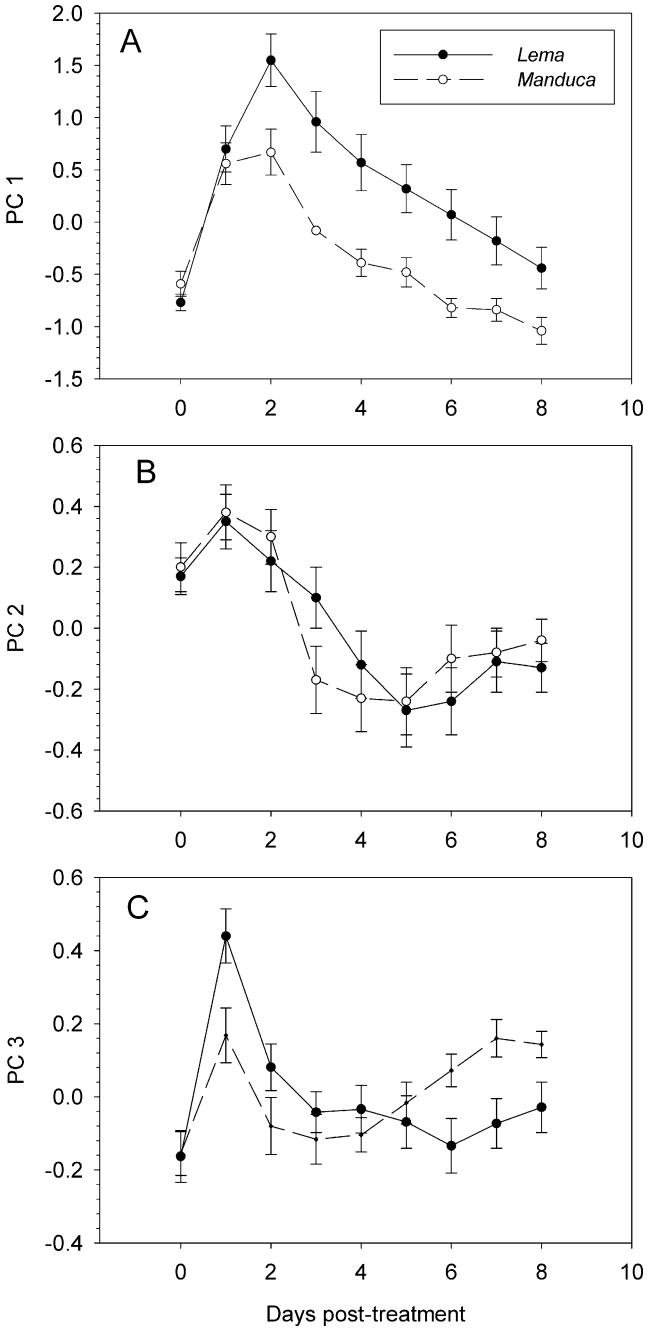


Fig. 3 Mean (± standard error) factor scores for principal components 1–3 for plants damaged either by larvae of *Lema daturaphila* or *Manduca sexta* for 24 hr between Day 0 and Day 1 as in Fig. 1a

ANCOVA on the scores for PC 2 showed significant variation among days ($F_{8,\ 506}$=9.95, P<0.001, Fig. 3b) and was due to the day by treatment interaction ($F_{8,\ 506}$=3.36, P<0.001) but not among induction treatments overall ($F_{1,\ 506}$=1.34, P=0.25). Scores for PC 2 were independent of the level of defoliation (test of covariate, $F_{1,\ 506}$=1.33, P=0.25). In general, plants produced blends relatively richer in HIPVs that loaded positively on PC 2 during the day of damage. Afterward, they produced blends relatively richer in HIPVs that loaded negatively on PC 2, principally TMTT, during the remainder of the experiment (Fig. 3b). Although the day by treatment interaction was statistically significant overall, on no day did the scores for PC 2 differ significantly among induction treatments. The closest difference to statistical significance occurred 3 d after induction (P= 0.074), in which the blend from plants induced by *L. daturaphila* was marginally richer in compounds with positive scores for PC 2 compared to plants induced by *M. sexta*.

ANCOVA on the factor scores for PC 3 also showed statistically significant variation over days ($F_{8,\ 506}$=9.79, P<0.001, Fig. 3c), and the day by treatment interaction ($F_{8,\ 506}$=4.33, P<0.001). There was no overall variation in PC scores between herbivore species ($F_{1,\ 506}$=0.00, P=0.99) but scores for PC 3 declined significantly with percent defoliation (test of covariate, $F_{1,\ 506}$=7.23, P=0.007). Apparently higher levels of damage were associated with higher proportions of (*E*)-β-caryophyllene and lower concentrations of most other compounds. Decomposing the day by treatment interaction showed that treatments differed only on the day of induction and the day following (P=0.003 and 0.03, respectively, Fig. 3c).

The individual compounds that contributed to the differences in PC 2 and PC 3 over time were revealed by comparing the temporal pattern of HIPV emission for the six compounds with the greatest absolute value for factor loadings on PC 2 and PC 3. For (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene DMNT, (*E*)-β-caryophyllene, and (*E,E*)-α-farnesene, peak concentrations were reached 1 d after herbivores were removed, and HIPV concentration declined regularly over the next 6 d (Fig. 4). By contrast, concentrations of TMTT persisted at near peak concentration for 3 d after insects were removed before declining. The persistence of TMTT during the middle of the observation period, while other HIPVs declined, accounted for the

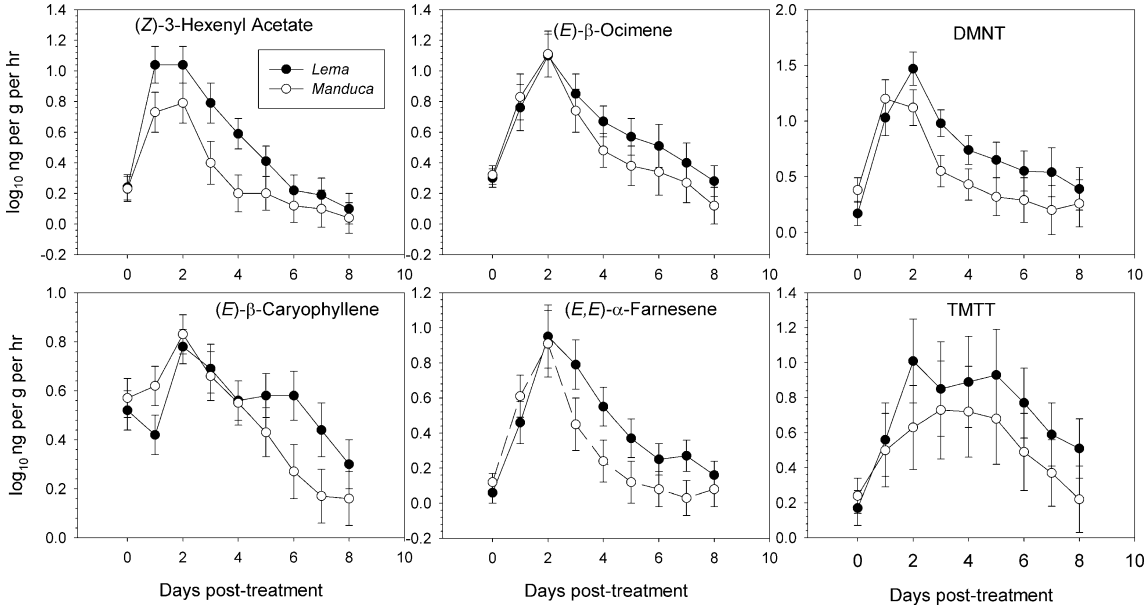


Fig. 4 Production of (*Z*)-3-hexenyl acetate, DMNT, (*E*)-β-ocimene, (*E*)-β caryophyllene, (*E,E*)-α-farnesene, and TMTT for plants damaged by *Lema daturaphila* or *Manduca sexta* for 24 hr between Day 0 and Day 1 as in Fig. 1a. Mean (± standard errors) in ng g^{-1} (dry wt.) leaf hr^{-1} are shown

change, with the composition of the blend becoming relatively richer in components loading negatively on PC 2 (i.e., TMTT) over time. The temporal pattern of variation in PC 3 is best reflected in the temporal pattern of variation in (*Z*)-3-hexenyl acetate (Fig. 4).

Lema, Tupiocoris, and Both A preliminary analysis on total HIPV production showed, once again, that HIPV production did not differ among plants to be assigned to the various experiments prior to induction ($F_{3,\ 58}$=0.51, *P*=0.67, Fig. 1b). HIPV production of control plants again did not differ among days ($F_{8,\ 545}$=0.70, *P*=0.69) but that for the other three treatments did differ among days (all $P \leq 0.02$). Highest average HIPV production of 198±72 ng HIPVs g^{-1} hr^{-1} occurred in the plants damaged by both *L. daturaphila* and *T. notatus*, 1 d after all insects were removed (Fig. 1b). Because the control plants also remained uninduced through the course of this experiment, they were removed from subsequent analyses to simplify the comparison of HIPVs produced by the different herbivore treatments. Mean leaf area removed by *L. daturaphila* was 5.06±0.9 cm^2, or 5.80±0.66% of available leaf tissue and did not differ between plants attacked by *L. daturaphila* or by *L. daturaphila* and *T. notatus*. In this experiment, the variation in total HIPV production was independent of the variation in herbivore damage ($F_{1,\ 330}$=0.96, *P*=0.33), probably because of the more consistent level of feeding compared to the first experiment, and there was no need to use defoliation as a covariate in subsequent analyses.

Results of the PCA analysis on HIPVs from insect-damaged plants showed that the first component (PC 1) accounted for 49% of the total variance, and PC 2 accounted for an additional 14.5%. PC 3 accounted for 10% of the total variance; the remaining principal components accounted for less than 10% each of the remaining variance and will not be considered in detail. All original variables except nerolidol had positive loadings on PC 1. The most abundant compounds were generally similar to those in the experiment comparing damage by *L. daturaphila* and *M. sexta* (Fig. 5a). In this experiment, PC 2 largely contrasted the abundance of (*E*)-β-caryophyllene with most of the rest of the HIPVs (Fig. 5a), and PC 3 independently contrasts plants producing high levels of TMTT with plants producing higher DMNT and (*Z*)-3-hexenyl acetate (Fig. 5b).

An analysis of variance on the factor scores for PC 1 showed significant differences among induction treatments overall ($F_{2,\ 445}$=11.06, *P*<0.001), over time ($F_{8,\ 445}$= 16.58, *P*<0.001), and scores for PC 1 also differed significantly due to the day by treatment interaction ($F_{16,\ 445}$=1.85, *P*<0.001, Fig. 6a). Scores for PC 1 did not differ significantly due to trichome genotype or any interactions involving trichome genotype (all $P \geq 0.16$). Decomposing the day by treatment interaction showed that PC 1 scores did not differ before insect damage ($F_{2,\ 445}$=0.81, *P*=0.45) but did differ among induction treatments starting the day of damage and continued to differ through the end of the experiment (all $P \leq 0.05$). On all days, plants damaged by both *Lema* and *Tupiocoris* emitted more HIPVs than did

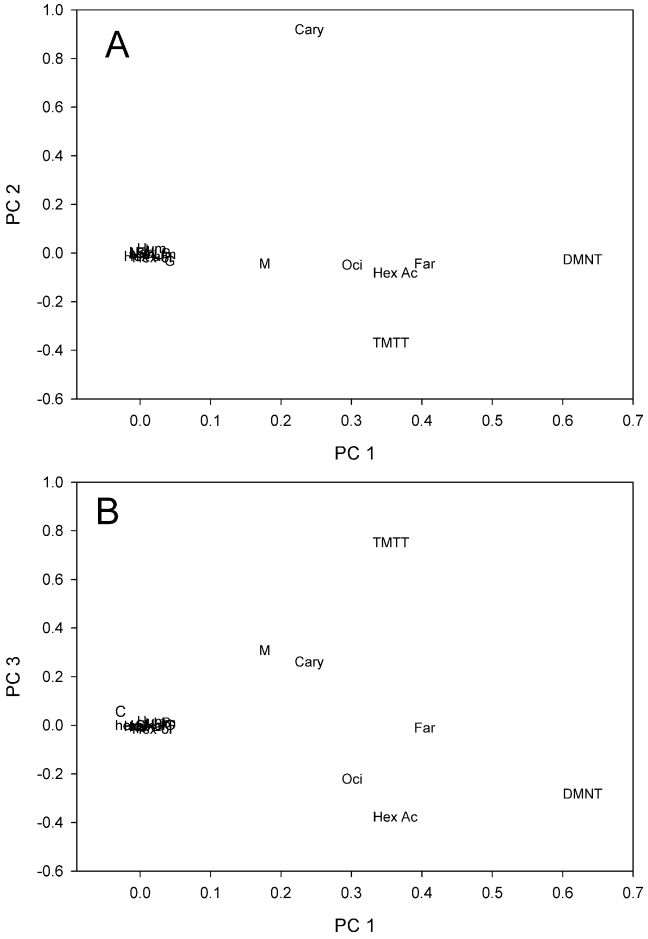


Fig. 5 Factor loadings for individual compounds for plants damaged by *Lema daturaphila, Tupiocoris notatus*, or both insect species simultaneously as in Fig. 1b. Plot **a**: PC1 and PC 2; plot **b**: PC1 and PC 3. Chemical abbreviations are as in Fig. 2

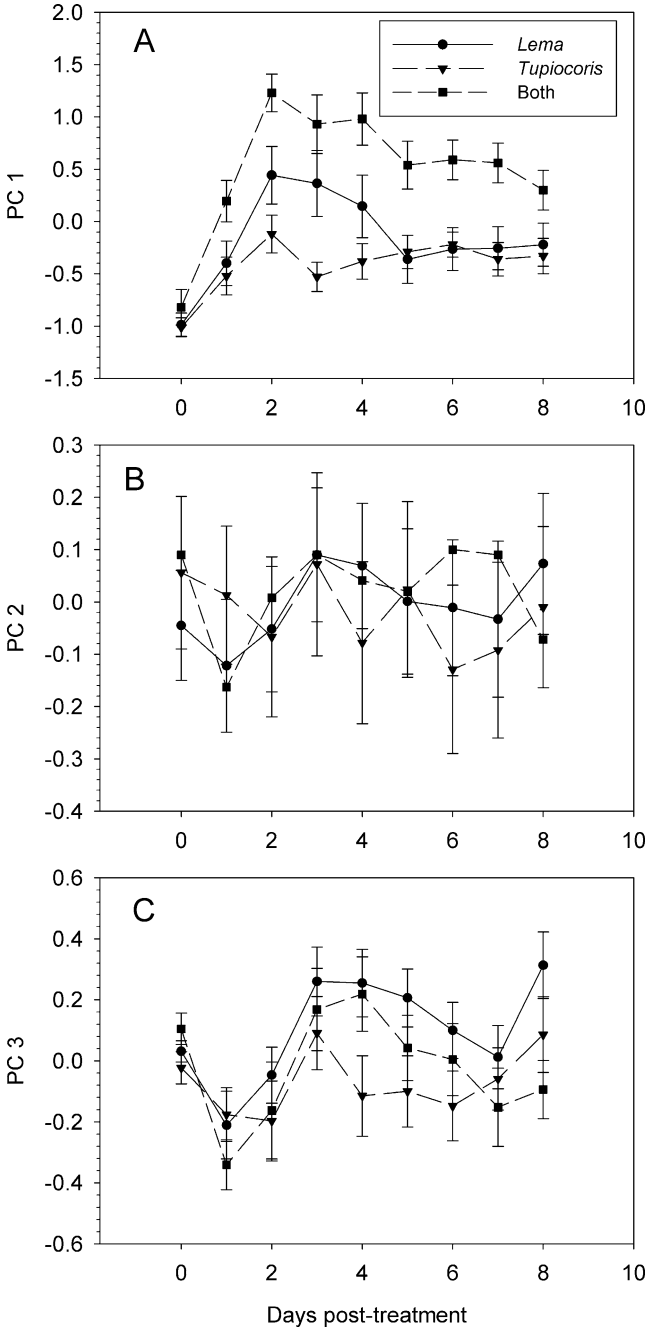


Fig. 6 Mean (± standard error) factor scores for principal components 1–3 for plants damaged either by *Lema daturaphila, Tupiocoris notatus,* or both insect species simultaneously as in Fig. 1b, for 24 hr between Day 0 and Day 1

plants damaged by either alone, and for all but the last 4 d, plants damaged by *Lema* emitted greater quantities of HIPVs than plants damaged by *Tupiocoris* (Fig. 6a).

For the plants damaged by both herbivores, the nonparametric analysis showed that the increased emission of plants damaged did not differ significantly from the null hypothesis of additive effects of damage by each herbivore species individually. Over the 160 observations in the full experiment (4 plants per block infested with both *L. daturaphila* and *T. notatus*, 5 blocks, and 8 d of observations), in 86 cases, plants produced lower total HIPV quantities than expected and in 74 cases they produced higher HIPV quantities than expected. These frequencies did not differ from the expected distribution of 80:80 (G_1=0.901, P=0.34). There also was no heterogeneity in the frequency distributions on different days (G_7=4.359, P=0.74).

ANOVA on the scores for PC 2, one index of the composition of the HIPV blend, showed no significant variation over time, treatment, or the time by treatment interaction (all $P \geq 0.14$, Fig. 6b). The scores for PC 3 differed over time ($F_{8,\ 445}$=8.54, P<0.001) and due to the day by treatment interaction ($F_{16,\ 445}$=1.94, P=0.016). Scores for PC 3 did not differ overall due to treatment (P=0.19), nor due to trichome genotype (P=0.15), nor due to any interaction including trichome genotype (all $P \geq 0.15$, Fig. 6c). Decomposing the day by treatment interaction showed that scores for PC 3 differed significantly only on

the last day of the experiment ($F_{2, 445}$=4.21, P=0.016). The largest difference was between plants damaged by *L. daturaphila* and plants damaged by both herbivores.

Individual Components The differences in PC 2 and PC 3 over time were revealed by comparing the temporal pattern of HIPV emission for the same 6 compounds as in Experiment # 1, but the temporal dynamics differed somewhat between the experiments. For (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, and DMNT, the highest concentrations were seen on the day after herbivores had been removed, as was the case in the first experiment. Also similar to the first experiment, peak concentrations of TMTT were somewhat delayed. In contrast to the first experiment, peak concentrations of (*E*)-β-caryophyllene and (*E,E*)-α-farnesene were not reached on the 1st d after insects were removed, but one to 2 d later, depending upon treatment (Fig. 7).

In summary, induction of plants by both *L. daturaphila* and *T. notatus* caused a greater emission of HIPVs than induction of plants by either herbivore species alone, but the increased emissions did not differ significantly from that predicted as the sum of the effects of each herbivore species' damage individually. There was no evidence that the differences in modes of feeding by *T. notatus* and *L. daturaphila* resulted in blends of different composition. The greatest source of variation overall was that due to the number of days since damage. The blends never differed consistently due to the differences in trichome genotype.

Discussion

The composition of HIPV blends from *D. wrightii* induced by feeding damage from *M. sexta* and *L. daturaphila,* or *L. daturaphila* and *T. notatus*, either alone or in combination, varied substantially with the number of days since damage, but there was no evidence that the HIPV blends characteristically and consistently differed between species of herbivore. Most of the induced monoterpenes [e.g., (*E*)-β-ocimene) and sesquiterpenes (e.g., (*E,E)*-α-farnesene, (*E*)-β-caryophyllene] reached peak emission rates within 1–3 d after the termination of plant damage, then declined over the next 4–6 d. In contrast, concentrations of the 16-carbon homosesquiterpene, TMTT, rose more slowly and persisted longer than the major mono- and sesquiterpenes. As a result, the HIPV blends became richer in TMTT in all treatments as the time since damage increased.

Temporal variation in HIPV production has been noted before in other systems. Commonly, C_6 green leaf volatiles are emitted from damaged leaves almost immediately after induction, and the emission of other compounds begins some time later. The amount of time between the emission of green leaf volatiles and other compounds varies among plant species. For example, Turlings et al. (1998) found significant temporal variation in the production of HIPVs in maize over a period of only 12 hr. In cotton, however, inducible terpene production peaked about a day after insect damage was terminated (Loughrin et al., 1994). In Brussels sprouts, 3 d of damage by 100 first- or second-instar caterpillars of *Pieris brassicae* was necessary to

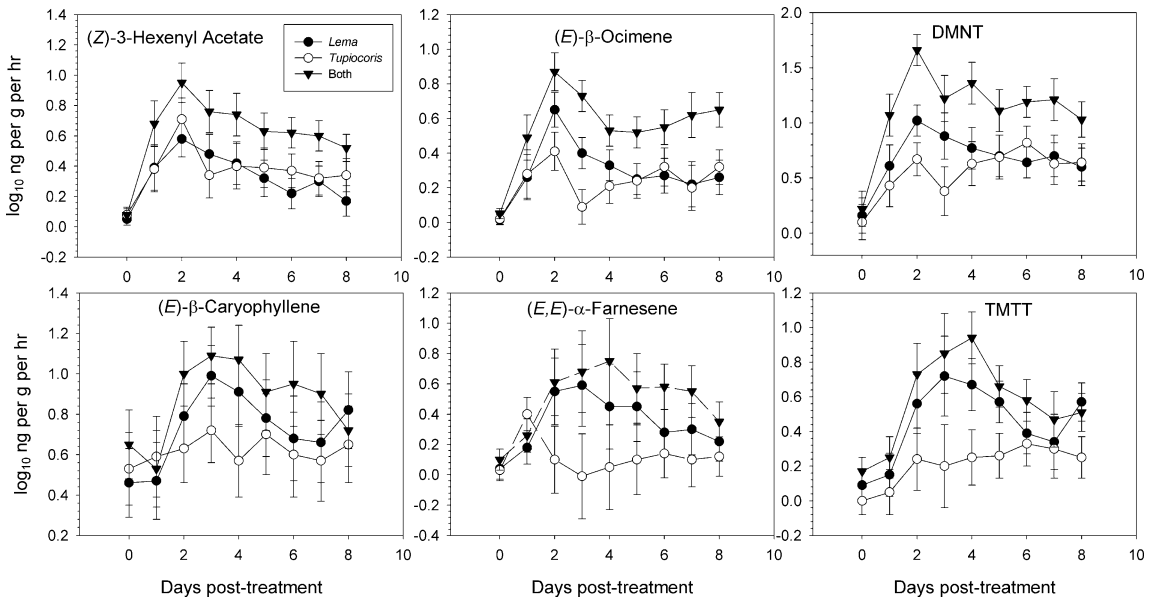


Fig. 7 Production of (*Z*)-3-hexenyl acetate, DMNT, (*E*)-β-ocimene, (*E*)-β caryophyllene, (*E,E*)-α-farnesene, and TMTT for plants damaged by *Lema daturaphila, Tupiocoris notatus*, or both insect species simultaneously as in Fig. 1b, for 24 hr between Day 0 and Day 1. Mean (± standard errors) in ng g^{-1} (dry wt.) leaf hr^{-1} are shown

induce sufficient HIPV production to attract its parasitoid *Cotesia glomerata*, and this response persisted for a day after insect herbivory ceased (Mattiacci et al., 2001). Similarly in *Brassica oleraceae,* plants subjected to 24 hr of herbivory or treated with JA remained attractive to parasitoids for 3 d after insects were removed and 5 d after JA treatment (Bruinsma et al., 2009).

Not only may total quantities vary temporally, but the production of individual components may follow independent time courses as well. In maize, DMNT was one of the first compounds produced, and the sesquiterpenes (*E*)-β-caryophyllene and (*E*)-β-farnesene were produced relatively later (Turlings et al., 1998). In tomato, TMTT and several terpenoids were induced within 3 hr of damage by *M. sexta,* but MeSA was not emitted until the 2nd d after damage (Farag and Pare, 2002). Different proportions of classes of volatile compounds also are emitted over a period from 1–37 hr in *Nicotiana attenuata*, with hexenyl esters predominating in the first 13 hr after damage and terpenoids in later time periods (Gaquerel et al., 2009).

Interestingly, different genotypes of plant species may exhibit different temporal profiles of HIPV emission; in one cultivar of maize, indole was emitted as early as DMNT, but in another, its peak emission was delayed to about the same time as the sesquiterpenes (Turlings et al., 1998). Thus, there are numerous precedents from other systems for the temporal variation seen in *D. wrightii*, not only in the total quantities of HIPVs, but also in the composition of the HIPV blends elicited by a single inducer. Such results suggest that the synthesis of different classes of compounds is regulated on different time scales following damage within species, but temporal pattern of regulation also can vary among species or genotypes within species.

Although the initiation of HIPV production in *D. wrightii* began on the day of damage and peaked 1–2 d later, the production of induced volatiles persisted for nearly a week. Such long relaxation times seen here and in other systems (Bruinsma et al., 2009) may complicate the efficient use of HIPVs as indicators of herbivores on plants because the HIPVs may be providing false information about the presence of actively-feeding herbivores (Puente et al., 2008a,b). One possible solution to this problem would be if the blend changed sufficiently over time that natural enemies could discriminate old damage from current damage. The solitary specialist parasitoid, *Cotesia vestalis*, for example, can make such discrimination on the basis of plant breakdown products that are emitted during herbivory but not after. The discrimination is imprecise because wasps still are attracted to plants with old damage when offered plants with old damage and plants with no damage (Kugimiya et al., 2010). The context-dependent nature of the responses of this parasitoid suggests that if there were natural selection on plants for the production of honest cues, then selection should favor the rapid cessation of HIPV emission when herbivores are no longer on those plants, rather than the continued emission of a blend of a different composition.

These experiments with *D. wrightii* in the laboratory, like most other laboratory experiments, greatly simplify how plants are attacked in nature. Under natural conditions, individual *D. wrightii* plants occur in patches that persist for several years (Hare and Elle, 2004). Plants quickly become colonized by overwintering adults of *L. daturaphila*, *T. notatus*, and other herbivores in the early spring, then suffer chronic herbivory throughout the growing season (Elle and Hare, 2000; Hare and Elle, 2002; Hare, 2010). Commonly, plants are infested with multiple life stages of *L. daturaphila* and other herbivorous insects throughout the season. It, therefore, is possible that damage inflicted by a single herbivore species for only a single day, as in this laboratory study, exaggerates the potential change in HIPVs over time compared to plants that are attacked under more natural conditions in the field.

Velvety plants infested with *T. notatus* were included in our experiments even though such infestations rarely occur in the field. We chose to include both trichome genotypes in order to avoid confounding herbivore species with trichome genotype when comparing the HIPV blends of sticky plants infested with *T. notatus* and *L. daturaphila* with velvety plants infested only with *L. daturaphila*¸ as is usually the case in the field. Despite the ability of feeding by *T. notatus* to suppress some jasmonate-mediated responses in other plant species (Heidel and Baldwin, 2004), we found no persistent differences in the HIPV production and composition of either plant genotype when infested with the combination of *T. notatus* and *L. daturaphila* compared to plants infested by *L. daturaphila* alone. One of our initial motivations to study the HIPV production of *D. wrightii* was to determine if predators like *G. pallens* might be able to recognize and avoid sticky plants, on which they are less effective predators (Gassmann and Hare, 2005). This might be possible if 1) the two trichome genotypes differed intrinsically in HIPV production, or 2) if the HIPV production of sticky plants was altered from that of velvety plants by the presence of *T. notatus* on sticky plants. Taken together with previous research (Hare, 2007), it appears that the HIPV blends of *D. wrightii* do not vary among trichome genotypes either intrinsically or when the trichome genotypes are attacked by *L. daturaphila* or *L. daturaphila* in combination with *T. notatus*. Moreover, sticky and velvety plants in the field do not differ in the composition of their HIPV blends even though the sticky plants were attacked by *T. notatus* in combination with *L. daturaphila*, whereas only the velvety plants were attacked by the latter species (Hare, 2010).

In systems in which HIPV blends are observed to vary with the inducing herbivore species, it may be a consequence of additional elicitors being associated with particular herbivores (Bruinsma et al., 2009; Peiffer and Felton, 2009). The evidence here suggests that there may be little difference in the response of *D. wrightii* to elicitors from different herbivore species, for there were no consistent, statistically different blends induced by the different herbivore species or combinations of species in the two experiments. Even if there were differences not detectable by the instrumentation, the biological significance of such differences may be slight, especially with regard to generalist predators like *G. pallens* that utilize all three herbivore species locally as prey. This conclusion is strengthened by the results of field experiments in other systems that show that predation rates of *G. pallens* can be increased when plant volatiles are augmented simply with a single HIPV component (James, 2003; Halitschke et al., 2008), suggesting that the precise composition of HIPV blends may be of less importance for *G. pallens* than for other natural enemies.

In summary, our original goals were to determine if three herbivores of *D. wrightii* produced HIPV blends that might differ consistently to possibly indicate the identity of the herbivore causing the damage. This was not the case. We also asked if simultaneous attack of plants by *T. notatus* and *L. daturaphila* might induce HIPV blends that differed qualitatively from those elicited by each insect alone and this also was not the case. The blends did not differ significantly and consistently in composition, rather, simultaneous damage by *T. notatus* and *L. daturaphila* simply elevated in an additive manner the HIPV production of plants with both herbivores over that induced by each species independently. Because the HIPV blends did not differ significantly among herbivore species or between trichome types for any combination of herbivores tested, this suggests that HIPV production by *D. wrightii* is more likely a general than specific indicator of damage by herbivores (Kessler and Baldwin, 2001). The absence of abundant specialist natural enemies of *M. sexta, L. daturaphila,* or *T. notatus* that might differentially respond to herbivore-specific HIPV blends may limit the potential for evolution of such herbivore-specific HIPV blends in *D. wrightii*.

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Differential Performance of a Specialist and Two Generalist Herbivores and Their Parasitoids on *Plantago lanceolata*

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Abstract The ability to cope with plant defense chemicals differs between specialist and generalist species. In this study, we examined the effects of the concentration of the two main iridoid glycosides (IGs) in *Plantago lanceolata*, aucubin and catalpol, on the performance of a specialist and two generalist herbivores and their respective endoparasitoids. Development of the specialist herbivore *Melitaea cinxia* was unaffected by the total leaf IG concentration in its host plant. By contrast, the generalist herbivores *Spodoptera exigua* and *Chrysodeixis chalcites* showed delayed larval and pupal development on plant genotypes with high leaf IG concentrations, respectively. This result is in line with the idea that specialist herbivores are better adapted to allelochemicals in host plants on which they are specialized. *Melitaea cinxia* experienced less post-diapause larval and pupal mortality on its local Finnish *P. lanceolata* than on Dutch genotypes. This could not be explained by differences in IG profiles, suggesting that *M. cinxia* has adapted in response to attributes of its local host plants other than to IG chemistry. Development of the specialist parasitoid *Cotesia melitaearum* was unaffected by IG variation in the diet of its host *M. cinxia,* a response that was concordant with that of its host. By contrast, the development time responses of the generalist parasitoids *Hyposoter didymator* and *Cotesia marginiventris* differed from those of their generalist hosts, *S. exigua* and *C. chalcites*. While their hosts developed slowly on high-IG genotypes, development time of *H. didymator* was unaffected. *Cotesia marginiventris* actually developed faster on hosts fed high-IG genotypes, although they then had short adult longevity. The faster development of *C. marginiventris* on hosts that ate high-IG genotypes is in line with the "immunocompromized host" hypothesis, emphasizing the potential negative effects of toxic allelochemicals on the host's immune response.

Key Words Chemical defense · *Chrysodeixis chalcites* · *Cotesia marginiventris* · *Cotesia melitaearum* · *Hyposoter didymator* · Immunocompromised host · Iridoid glycosides · *Melitaea cinxia* · Multitrophic interactions · *Spodoptera exigua*

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Introduction

Plants have evolved a wide array of direct defenses against herbivores and diseases. Direct defenses include morphological traits (Cooper and Owen-Smith, 1986), and chemical toxins, repellents, or digestibility reducers (Fraenkel, 1959; Ehrlich and Raven, 1964). These defenses not only affect the performance of herbivores

but also can have positive or negative effects on natural enemies of herbivores. Positive effects of chemical defense in the herbivore's diet on natural enemies can arise in several ways. For instance, allelochemicals may slow down the growth rate of the herbivore, extending the time window during which the herbivore is vulnerable to parasitism or predation by its natural enemies (Clancy and Price, 1987). Toxic allelochemicals also may weaken host immune responses, making them more susceptible to parasitation or predation (Nappi, 1975; Smilanich et al., 2009). On the other hand, allelochemicals can have negative effects on the growth, development, or survival of predators and parasitoids (Gunasena et al., 1990; Paradise and Stamp, 1993; Harvey et al., 2007b). For instance, hosts may actively sequester these compounds in their haemolymph where they can again function as defense (Bowers, 1980, 1981; Camara, 1997b; Nishida, 2002).

The ability of herbivores to cope with plant allelochemicals varies among species that differ in their dietary range. Polyphagous species (generalists) feed on many species in a range of plant families (Bowers and Puttick, 1988; Agrawal, 2000). Even though many herbivores are polyphagous at the species level, they are not necessarily so at the population or individual level since species can be comprised of populations or individuals that are specialized on a few or even a single food plant (Singer, 2008). Some studies support the idea that detoxification systems of generalists are capable of accepting a more structurally diverse array of compounds than those of specialists (Li et al., 2004) at the expense of a less efficient detoxification of individual compounds (Johnson, 1999). Oligophagous species (specialists) often are physiologically adapted to the nutritional and secondary chemistry of particular plant species (Renwick et al., 2001; Cornell and Hawkins, 2003). They may use the allelochemicals to which they have adapted as host-finding cues and feeding stimulants (Rhoades, 1979; Bowers, 1981). A number of specialist herbivores sequester these substances as defense against their own natural enemies (Bowers, 1981), which requires physiological or morphological adaptations to prevent autotoxicity (Duffey, 1980).

The division between generalists and specialists also can be made for predators and parasitoids of herbivores. Allelochemicals present in herbivores, or in the diet of an herbivore, can reduce the performance of generalist predators and parasitoids (e.g., Duffey et al., 1986; Gunasena et al., 1990). Generalist parasitoids may attack a wide range of herbivorous host species that feed on plants with different defensive chemistry. These parasitoids may suffer during development if their hosts are able to sequester or accumulate plant derived compounds, to which they are not adapted (Barbosa, 1988; Barbosa et al., 1991; Harvey et al., 2005). In contrast, specialized natural enemies, such as many endoparasitoid wasps, are restricted to attacking only one or a few related host species, which generally feed on closely related plants (Godfray, 1994; Quicke, 1997). These parasitoids may be adapted to the limited set of chemicals to which they are exposed (Harvey et al., 2005), allowing them to use hosts that sequester specific plant chemical defenses (Barbosa et al., 1986; Sznajder and Harvey, 2003; Harvey et al., 2007a,b).

The types and concentrations of defensive substances found in plant tissues vary greatly among plant species as well as among individuals of the same species (Denno and McClure, 1983; Nieminen et al., 2003). In this study, we used genotypes of ribwort plantain [*Plantago lanceolata* L. (Plantaginaceae)] that differ in constitutive concentrations of two iridoid glycosides (IGs), aucubin and catalpol, to study the effects of allelochemical variation on specialist and generalist herbivore-parasitoid associations. Iridoid glycosides are monoterpenoids. Aucubin is the biosynthetic precursor of catalpol (Damtoft et al., 1983). The concentrations of IGs in *P. lanceolata* show large natural variation among individuals, and within individuals over time (Bowers and Stamp, 1992; Darrow and Bowers, 1997; Barton, 2007). Aucubin, and to a greater extent catalpol, are toxic or deterrent to generalists (Puttick and Bowers, 1988; Bowers, 1991). At the same time, these compounds serve as feeding and oviposition stimulants for some specialist herbivores (Bowers, 1984; Pereyra and Bowers, 1988; Nieminen et al., 2003; Reudler Talsma et al., 2008). Larvae and adults of the specialist butterflies *Melitaea cinxia* (Lepidoptera: Nymphalidae) and *Junonia coenia* (Lepidoptera: Nymphalidae) sequester aucubin and catalpol (Bowers and Collinge, 1992; Suomi et al., 2001, 2003). Larvae of *J. coenia* do this more efficiently than polyphagous insect herbivores (Lampert and Bowers, 2010), and they sequester catalpol more efficiently than aucubin (Bowers and Collinge, 1992). The latter is also observed for larvae of *M. cinxia* and *Parasemia plantaginis* (Lepidoptera: Arctiidae) (Reudler Talsma, unpublished). Only few studies have assessed the effect of these compounds on preference or performance of parasitoids, using dipteran (Mallampalli et al., 1996) or hymenopteran parasitoids (Nieminen et al., 2003; Harvey et al., 2005; Lampert et al., 2010). Our study extends that of Harvey et al. (2005) by including two generalist herbivores, each with two generalist parasitoids, to investigate whether effects of IG are consistent across these generalist systems, and compares them with a specialist system. Moreover, we include a series of plant genotypes that span a wide range of mean IG concentrations in order to gain quantitative insight into associations between plant IGs and insect development parameters.

We address the following questions: (1) Is the performance of herbivores and their parasitoids affected

by the concentrations of the iridoid glycosides aucubin and catalpol in *P. lanceolata*? (2) Are there consistent differences in the effect of plant iridoid glycosides on the performance of specialist vs. generalist herbivores and parasitoids?

Methods and Material

Plants

Plantago lanceolata is a perennial plant with a worldwide distribution (Sagar and Harper, 1964). In natural populations, IG levels range from undetectable to ca. 12% of the plant dry weight (Bowers et al., 1992; Fajer et al., 1992). There generally is no correlation between IG concentrations (aucubin or catalpol) and tissue nutrient concentrations (Marak et al., 2000; Reudler Talsma, 2007). Plants used for our experiments came from two sources. Thirteen genotypes, used in all experiments, were derived from an artificial selection experiment in which lines were selected for high and low leaf IG concentrations for four generations, originating from Dutch *P. lanceolata* (Marak et al., 2000). These genotypes, each originating from a different half-sib family, were clonally propagated (Wu and Antonovics, 1975) in order to generate sufficient leaf material of each genotype. For experiments involving the specialist butterfly *M. cinxia* and its specialist endoparasitoid *Cotesia melitaearum,* (Hymenoptera: Braconidae), we augmented this set of genotypes with three additional genotypes from the selection experiment, and ten genotypes collected from Åland, Finland [where the insects originated (see below)]. We tested the effects of quantitative variation in allelochemicals in the plants on the development of six different insect species: two generalist herbivores, two generalist endoparasitoids reared on each of these generalist herbivores, and a specialist herbivore and its specialist endoparasitoid.

Herbivores

Generalists The beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae), is polyphagous, and a serious pest of many crops worldwide. Females lay several clusters of 50–100 eggs that hatch within 2–3 d. The larvae normally have five instars (the first three are gregarious), and the entire life cycle is about 4–5 weeks with several generations per year (Wilson, 1934; Tingle and Mitchell, 1977). Cultures of *S. exigua* were established from eggs originating from a laboratory culture maintained on artificial diet at the Department of Virology at Wageningen University, the Netherlands.

The golden twin spot, *Chrysodeixis chalcites* (Lepidoptera: Noctuidae) is native to Central and Southern Europe, the Canary Islands, and Africa. Like *S. exigua*, it is a polyphagous crop pest. Eggs are laid singly or in small groups and hatch in 4–9 d. The larvae normally complete 6 instars and the entire life cycle is completed in about 5–7 weeks (Goodey, 1991). *Chrysodeixis chalcites* cultures were established from individuals collected from a garden in Nijmegen, the Netherlands.

Cultures of both *S. exigua* and *C. chalcites* were kept as caterpillars in plastic Petri dishes on artificial diet (Elzinga, 2002) at 25°C and 16:8 h L:D. When caterpillars were in their final instar, they were placed in plastic containers (15×10×10 cm) with diet and vermiculite into which they pupated. In order to get larvae for the experiment, adult moths were placed in a cage (40×50×65 cm) in a climate room at 25°C, 55% RH and 16:8 h L:D with honey water (1:1) and one *P. lanceolata* plant, that both noctuid species readily accepted for oviposition. Newly hatched caterpillars were collected from this cage and reared in Petri dishes (8 cm) on artificial diet until they reached their 3rd instar, after which they were used in the performance studies. The early growth on artificial diet was done in order to have sufficient numbers of caterpillars for the experiments, as their 1st instars survive better on artificial diet than on *P. lanceolata* diet (personal observation). Note that since the artificial diet contains no IGs, the effect of IG on larval and parasitoid performance is limited to what was ingested as later instars.

Specialist The Glanville fritillary butterfly, *M. cinxia,* inhabits open grasslands in parts of Europe and temperate Asia. It has one generation per year in most of its range. A female lays several large egg clusters (150–200) on plants in the genera *Plantago* and *Veronica* (Plantaginaceae) (Kuussaari et al., 2004). Larvae spin a communal web and feed gregariously throughout most of their development. Higher levels of IGs in the diet can increase the rate of development (Harvey et al., 2005; Saastamoinen et al., 2007). Furthermore, IGs are sequestered by the larvae (Suomi et al., 2001, 2003), and plants with higher IG levels are preferred for oviposition (Nieminen et al., 2003; Reudler Talsma et al., 2008). *Melitaea cinxia* caterpillars used for these experiments were the offspring of laboratory reared butterflies from Åland, SW Finland. The caterpillars were fed field collected *P. lanceolata* leaves until their 3rd instar when they were used for the experiment. Note that since rearing of *M. cinxia* on specific plant genotypes did not start until they reached their 3rd instar, assessment of effects of IG concentration on larval and parasitoid performance is limited to what was ingested as later instars.

Parasitoids

Generalists Hyposoter didymator (Thunberg, 1822) (Hymenoptera: Ichneumonidae) is a generalist solitary endoparasi-

toid of caterpillars of many species in the large Lepidoptera family Noctuidae (Bar et al., 1979; Ingram, 1981; Vinson, 1990; Figueiredo et al., 2000; Schneider et al., 2003). This species is a tissue feeder, so the developing wasp larva consumes the entire host. *Hyposoter didymator* adults were obtained from a laboratory colony that was started in 1993 with wasps collected from parasitized *Spodoptera littoralis* in the Cordoba, Spain. For 4 years, they were reared on *S. frugiperda* in the Laboratoire de Pathologie Comparée at INRA UMII, Saint-Christol-lèz-Alés, France. Adults used in the experiment were reared on *S. exigua*. In order to extend longevity of the adult wasps, they were kept at 10°C and 16:8 h L:D.

Cotesia marginiventris (Cresson, 1865) (Hymenoptera: Braconidae) also is a generalist solitary endoparasitoid that attacks Noctuidae (Krombein et al., 1979). As other *Cotesia*, the larva feeds primarily on host haemolymph and fat body during its development, and it does not consume the entire host (Sznajder and Harvey, 2003). *Cotesia marginiventris* were obtained from a colony maintained at Nijmegen University, The Netherlands, where they were reared on *S. exigua.* Adults were kept at 10°C and 16:8 h L:D and were constantly provided with honey and water.

Specialist Cotesia melitaearum agg. is a complex of cryptic species specialized on *Melitaea* or *Euphydryas* butterflies (Kankare and Shaw, 2004). The species of *C. melitaearum* agg. used in this study is restricted to the host *M. cinxia* (Kankare et al., 2005). The adult female parasitoid lays broods of 1–40 eggs (depending on host size) in all instars of *M. cinxia* larvae. We used *C. melitaearum* from Åland, SW Finland, where the wasp has 2–3 generations per year (Lei et al., 1997). For the experiment, laboratory reared *M. cinxia* caterpillars were put on potted plants that were placed in natural populations in Åland, where they were parasitized by naturally occurring *C. melitaearum.*

Experimental Design

Generalists Third instars of the two generalist herbivores, *S. exigua* and *C. chalcites,* were randomly assigned to one of three treatments: unparasitized (control), parasitized by *C. marginiventris*, or parasitized by *H. didymator.* For parasitism, a 3rd instar caterpillar was offered to a female parasitoid, and oviposition was observed as a single insertion of the ovipositor. Although the parasitoids attack all larval instars of *S. exigua* and *C. chalcites*, previous studies have shown that *H. didymator* has the highest reproductive success when it parasitizes 3rd instar hosts (Reudler Talsma et al., 2007). For each *P. lanceolata* genotype, 16–20 parasitized and 16–20 unparasitized caterpillars of each herbivore species were reared individually on freshly excised leaves in Petri dishes (55 mm diam) in a growth cabinet at 25°C, 16:8 h L:D and 70% RH. The total number of larvae included in the experiment was 451. Leaves were refreshed every day. This was done using young to intermediate-aged leaves from at least five different plant individuals per genotype. Leaves were collected and then pooled per genotype and distributed across the larvae assigned to that genotype. Due to insufficient leaf production by some of the genotypes, not all genotypes were represented in every treatment combination. Emerged adults (both butterflies and parasitoids) were provided with water and kept at 25°C, 16:8 h L:D; 70% RH. The following fitness correlates were recorded for the unparasitized caterpillars: larval development time (the number of days from the 3rd instar, when the experiment started, until pupation), pupal development time, pupal mass, pupal survival until adulthood, adult mass, and adult longevity. The same parameters were recorded for the parasitoids, except that larval development time was recorded from the day of parasitism until the larva emerged from the host, and pupal development time was the number of days between larval emergence and adult wasp eclosion. Pupal mass of parasitoids includes the silk of the cocoon.

Specialist The experiments with the specialist *M. cinxia* and its parasitoid *C. melitaearum* were undertaken in the field to allow for natural parasitism. Gregarious larval groups of *M. cinxia* were placed on intact potted plants in field locations where the parasitoid *C. melitaearum* is known to occur. Before putting the larvae on the plant, they were fed fresh leaves collected from *P. lanceolata* in the field. Groups of forty 3rd-instar caterpillars were placed onto single potted plants of 26 different genotypes of *P. lanceolata* (16 originating from the Netherlands, including the 13 genotypes used in the generalist experiments, and 10 originating from Åland, Finland). Five replicate plants of Dutch genotypes and three replicate plants of Finnish genotypes were used. Three plants were lost, so in total we used 107 plants and 4,280 caterpillars. Each pot was covered by a mesh cloth that prevented the caterpillars from escaping, but which allowed *C. melitaearum* to enter. Caterpillars were left in the field for 3 weeks, during which time their host plant was replaced with the same genotype if it was defoliated. After 3 weeks, all caterpillars had molted to their 5th (diapause) instar. The 2,728 recovered caterpillars then were removed from the plants, and a random subsample of caterpillars from each plant (1,501 in total) was placed in a separate plastic container in a root cellar in Åland, where the larvae spent the winter in diapause. The following spring their diapause was broken and the larvae were fed fresh leaves from the same genotype on which

they had fed the previous summer. Caterpillars were reared until pupation after the 7th larval instar, or larval parasitoid emergence. Post-diapause, the same parameters were measured as in the experiment with the generalists, except that larval development time was determined as the number of days between the breaking of diapauses, and emergence; adult longevity was not measured. Additionally we measured the survival of the larvae until diapause, and the number that survived overwintering. Because the hosts were parasitized naturally, rather than under visual observation in the laboratory, only a small fraction of the larvae was found to be parasitized (the natural rate of parasitism is low) (van Nouhuys and Punju, 2010). This approach allowed us to compare both the performance parameters and the rate of parasitism of larvae on the different genotypes of *P. lanceolata.*

Chemical Analyses

Collection of Leaf Material In the experiments with generalist herbivores and parasitoids, one mixed leaf sample per genotype was collected at three 4 weeks intervals, for analysis of leaf IG concentrations. The first two time points were during the growth period of the larvae. IG estimates from these two time points were averaged per genotype and used in tests of associations with insect performance parameters. Measurements from the third time point were used only to check for consistency of genotype differences over a longer time period. Each leaf sample consisted of the young and intermediate-aged leaves of at least 5 plants of the same genotype, as in the feeding trials. For the experiments with the *specialist* herbivore and parasitoid, leaves were sampled both pre- and post-diapause. From each plant, the 3rd and 4th fully grown leaf was collected before putting the pre-diapause caterpillars on them in the field. When a plant was completely defoliated and replaced by another of the same genotype, we also took the 3rd and 4th fully grown leaf from the second plant, and averaged their IG levels. Concentrations then were averaged per genotype and used in analyses of parasitism and survival until the breaking of diapause. After diapause, we took two subsamples of the leaves that were fed to the caterpillars per genotype, one early and one late during the remaining ca. 6 weeks development time of *M. cinxia*. The averages of these concentrations per genotype were then used to test for associations with post-diapause performance parameters.

Leaf IG Analysis These methods follow Marak et al. (2003). In brief, all leaves were freeze dried and ground to a fine powder with a ball mill (Retsch, type MM 301, Retsch GmbH and Co., Haan, Germany). Finely ground dry material from the leaves (25 mg) was extracted in 10 ml of 70% MeOH and was shaken overnight (15°C/100RPM). The crude extract was filtered through Whatman #4 filter paper and the filtrate was diluted ten times with Milli-Q water. Concentrations of the IGs aucubin and catalpol were analyzed by HPLC using a Bio-Lc (Dionex, Sunnyvale, USA) equipped with a GP40 gradient pump, a Carbopac PA 1 guard (4×50 mm) and analytical column (4×250 mm), and an ED40 electrochemical detector for pulsed amperimetric detection (PAD). NaOH (1M) and Milli-Q water were used as eluents (10:90%, 1 ml/min). Retention times were 3.25 min and 4.40 min for aucubin and catalpol, respectively. Concentrations were analyzed using Chromeleon version 6.60 (Dionex Corp., Sunneyval, USA). A standard concentration series of aucubin and catalpol of 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 ppm was used for calibration.

Statistical Analysis

For the 13 plant genotypes that were included in both the generalist and specialist performance trials, we used ANOVA to analyze differences in IG between plant genotypes, between experiments (greenhouse-grown plants for generalists, field-grown plants for specialists), and their interaction. IG values were square-root transformed to meet assumptions of normality. In addition we used ANOVA to test whether there were differences in leaf IG concentrations between Dutch (artificially selected) and Finnish (collected from the wild) genotypes among the field grown plants.

Pearson correlations were used to test for associations between mean values for development parameters of the insects on the different plant genotypes and the mean IG concentration of those genotypes. The latter were based on the leaf samples collected during the corresponding experiment (greenhouse, field pre-diapause, or field post-diapause). For the generalist species, we analyzed larval and pupal development time, pupal and adult weight, and adult longevity. For the specialist,s the last two parameters were not measured. In addition, logistic regression was used to test whether mean larval and pupal survival on plant genotypes was affected by mean genotype IG concentration and whether parasitism of *M. cinxia* larvae by *C. melitaearum* was affected by mean genotype IG concentration. Since Dutch and Finnish genotypes appeared to differ in their IG profiles (catalpol and catalpol-to-total IG ratio, see results), we used ANCOVA to test whether genotype mean performance of specialists differed between genotypes from Finnish and Dutch origin, and whether effects of genotype origin were still significant if genotype mean catalpol or the ratio of catalpol to the total IG concentration [catalpol/(aucubin+catalpol)] were included as a covariate. All

statistical analyses were performed using SPSS for Windows Release 16.0.2.

Results

Iridoid Glycoside Variation Among Plant Genotypes

Genotype mean leaf IG concentrations of the 13 Dutch genotypes that were included in all performance studies varied circa six-fold, ranging from 1.28±0.21 to 7.35±0.90 (% leaf dry weight ± SE). The differences in leaf IG concentration among this set of genotypes were highly significant ($F[12,102]$=11.03, P<0.001). The same was true for the constituent components aucubin ($F[12,102]$=10.61, P<0.001) and catalpol ($F[12,102]$=15.47, P<0.001) that showed ranges of among-genotype variation similar to those observed for total IG concentration. IG concentrations were lower in the greenhouse-grown plants (generalist performance study, Fig. 1a, ranging from 0.83±0.09 to 6.41±1.02) ($F[1,102]$=13.16, P<0.001) than in field-grown plants (specialist performance study, Fig. 1b, ranging from 1.47±0.26 to 8.63±0.26), but the ranking of the genotypes was maintained under both conditions (interaction $F[12,102]$=1.10, P=0.36). Mean leaf IG concentrations of the additional genotypes used in the specialist performance study all fell within the range of the 13 genotypes that were shared between the experiments.

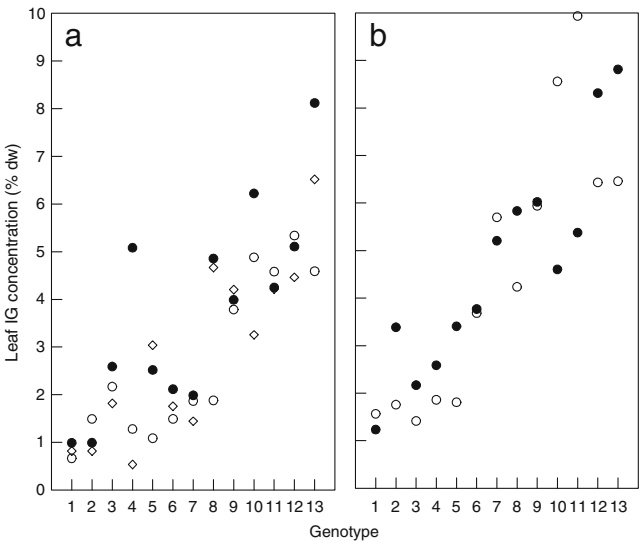


Fig. 1 Within- and between genotype variation in leaf iridoid glycoside (IG) concentrations in 13 *Plantago lanceolata* genotypes, ranked according to increasing genotype mean IG. (**a**) IG values from mixed leaf samples of each genotype taken at 4-wk intervals, two times during (*circles*) and one time after (*diamond*) the generalist feeding trials. (**b**) Mean values per genotype from samples taken before (*open circles*) and after diapause of specialists (*closed circles*), 10 months later

The mean IG values of the Finnish genotypes did not differ from the Dutch ones ($F[1,24]$=0.24, P=0.63), but they contained more catalpol (2.01±0.28 vs. 1.09±0.28, $F[1,24]$=9.82, P=0.004), resulting in a higher contribution of catalpol to the total IG level in Finnish (41.9%) than in the Dutch genotypes (31.7%). Since variation in IG concentrations between genotypes was large compared to the temporal variation within genotypes at the scale of weeks (Fig. 1a) or even months (Fig. 1b), and since the ranking of genotypes with respect to their leaf IG concentrations was fairly consistent over time (Fig. 1a and b), we are confident that even though we did not intensively sample IGs during larval development, the genotype mean IG values that we used provide a reasonable estimate of differences in IG levels experienced by the insects during the experiment.

Effects of Iridoid Glycosides on Specialist and Generalist Herbivores

Generalists Larvae of the generalist herbivore *C. chalcites* developed slowly on plant genotypes with high IG concentrations (Table 1). For instance, an increase in the IG concentration from 1% to 6%, the range spanned by the genotypes, caused a 20.0% (4.2 day) delay in larval development time (Fig. 2a). Development of the other generalist herbivore, *S. exigua,* also was slower on genotypes with higher IG concentrations, but in this species it was the pupal development that was delayed (Table 1). An increase in the average IG concentration from 1% to 6% of leaf dry weight caused a 9.9% (0.82 day) delay in pupal development (Fig. 2b). Adult longevity of *S. exigua* also was negatively affected by high leaf IG concentrations in its diet (Fig. 3a; r=−0.642, P=0.03). An increase in IG concentration from 1% to 6% of leaf dry weight reduced longevity by 59% (5.5 days). Adult longevity of the other generalist herbivore, *C. chalcites,* was independent of the concentration of IG in its diet (r=−0.102, P=0.77). Pupal weight, pupal survival, and adult weight of the generalist herbivores were unaffected by the leaf IG concentrations in their diet. This does not mean that these parameters were not affected by host plant genotype. For instance, pupal mass was strongly affected by host plant genotype in both *C. chalcites* ($F[10,88]$=12.81, P<0.001) and *S. exigua* ($F[12,90]$=3.94, P<0.001), but apparently these effects were not mediated by differences in the concentrations of IGs in these plant genotypes.

Specialist Roughly half (51.6%) of the caterpillars of the specialist *M. cinxia* from the 26 genotypes in the field and that had been randomly selected for further study survived diapause over winter. This is on par with the natural rate of overwintering survival (van Nouhuys et al., 2003). Their

Table 1 Correlations between mean leaf concentrations of iridoid glycosides and mean performance (larval development time, cocoon weight and pupal development time) of insect herbivores and their parasitoids on genotypes of *Plantago lanceolata*

Herbivore	Parasitoid	*N*	Larval development time	Cocoon weight	*N*	Pupal development time
Chrysodeixis chalcites		11	+0.684**	−0.434	11	−0.444
	Cotesia marginiventris	10	−0.643**	+0.568*	7	−0.500
	Hyposoter didymator	11	−0.050	−0.000	11	+0.219
Spodoptera exigua		13	−0.035	−0.308	11	+0.726**
	Cotesia marginiventris	13	−0.232	+0.214	13	−0.590**
	Hyposoter didymator	7	−0.466	−0.286	4	+0.093
Melitaea cinxia		21	+0.220	−0.047	11	−0.263
	Cotesia melitaearum	8	+0.198	−0.082	6	+0.133

N = Number of *P. lanceolata* genotypes included in the analysis
*$P<0.10$; **$P<0.05$

probability of survival was significantly lower when they originated from plant genotypes with high levels of catalpol before diapause (Fig. 4A, logistic regression, *Wald* $\chi^2[1]=8.23$, $P=0.004$). An increase in leaf catalpol concentration from 0.5% to 3.0%, the range encompassed by the genotypes, was associated with a decrease in survival from 59% to 37%. Survival of diapausing larvae was lower when they had been raised on Finnish (Fig. 4a, open symbols) than on Dutch genotypes (Fig. 4a, closed symbols) before diapause (*Wald* $\chi^2[1]=4.50$, $P=0.03$). This difference could be explained by the higher catalpol concentrations in Finnish genotypes: effect of genotype origin (Finnish vs. Dutch) was no longer significant in a multiple logistic regression in which both genotype origin and genotype mean catalpol concentration were entered, whereas the latter effect was still significant. None of the post-diapause developmental parameters of *M. cinxia* were significantly associated with post-diapause levels of IGs in the herbivore's diet (Table 1), except for a faster development of post-diapause larvae on genotypes with a higher catalpol-to-total IG ratio ($r=-0.504$, $P=0.02$). Consequently, post-diapause larval development of *M. cinxia* was

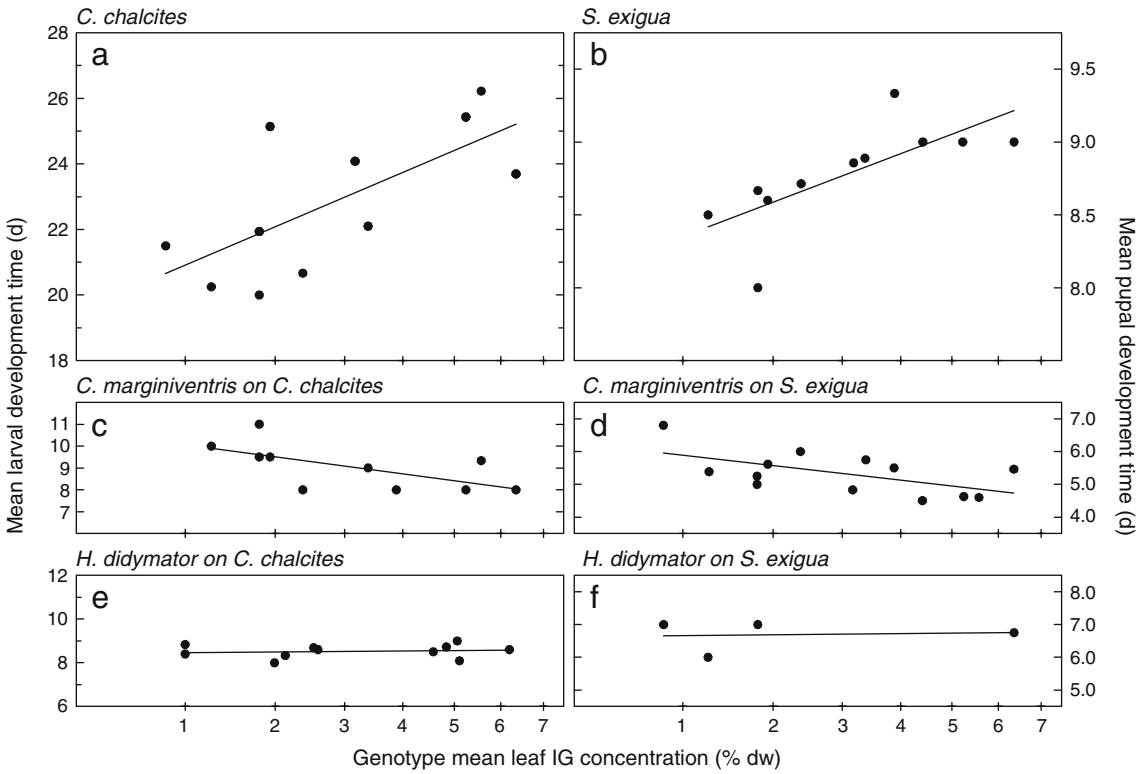


Fig. 2 Development time of generalist herbivores and parasitoids as a function of genotype mean leaf iridoid glycoside (IG) concentration in the food plant *Plantago lanceolata*. *Left panels*: mean larval development time of *Chrysodeixis chalcites* (**a**) and its parasitoids *Cotesia marginiventris* (**c**) and *Hyposoter didymator* (**e**). *Right panels*: mean pupal development time of *Spodoptera exigua* (**b**) and the same two parasitoids (**d**, **f**). Note the square-root scale of the X-axis

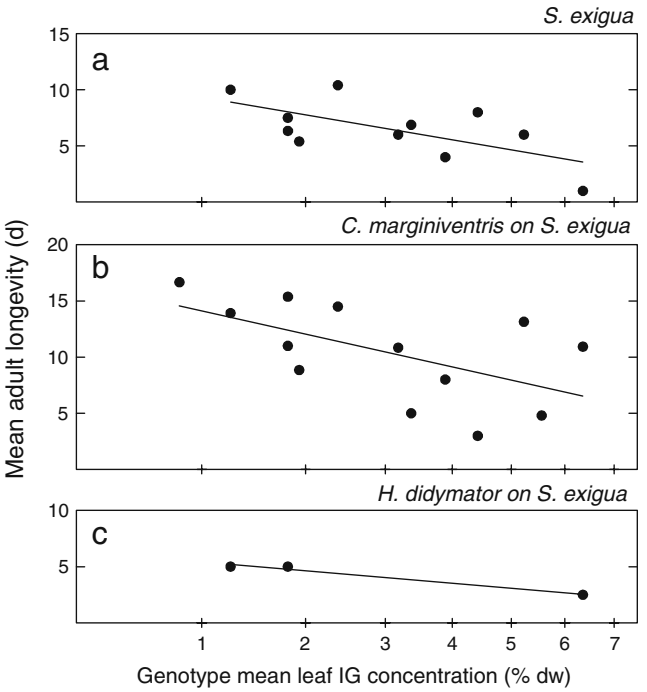


Fig. 3 Mean adult longevity of the generalist herbivore *Spodoptera exigua* (**a**) and its parasitoids *Cotesia marginiventris* (**b**) and *Hyposoter didymator* (**c**) as a function of genotype mean leaf iridoid glycoside (IG) concentration in the food plant *Plantago lanceolata*. Note the square-root scale of the X-axis

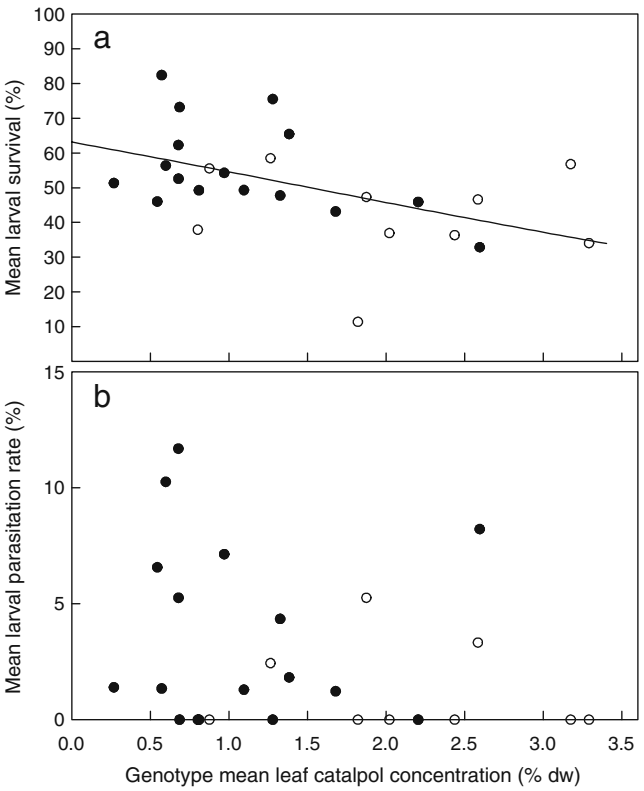


Fig. 4 (**a**) Mean survival of diapausing larvae of the specialist herbivore *Melitaea cinxia* and (**b**) Mean parasitism rate of *M. cinxia* by *Cotesia melitaearum* as a function of genotype mean leaf catalpol concentration in the food plant *Plantago lanceolata*. *Closed circles*: Dutch plant genotypes; *open circles*: Finnish plant genotypes. *Lines* are fitted values from logistic regressions based on data from all genotypes

faster on Finnish genotypes, that on average had a high catalpol-to-total IG ratio, than on Dutch genotypes (46.0±1.8 vs. 38.9±1.9 days, $F[1,20]=5.50$, $P=0.03$).

Additional analyses confirmed that the differences in post-diapause larval development time on Finnish vs. Dutch genotypes could be partly explained by differences in their IG profiles: when catalpol-to-total IG ratio was entered as a covariate in analyses of genotype origin (Finnish vs. Dutch) on larval development time, the significant effect of catalpol-to-total IG concentration remained ($F[1,19]=6.16$, $P=0.02$), whereas genotype origin (Finnish vs. Dutch) could explain only little additional variation ($F[1,19]=3.36$, $P=0.08$). This was not the case for post-diapause survival of *M. cinxia* larvae on Finnish vs. Dutch genotypes. Post-diapause survival until pupation was three times higher on Finnish than on Dutch genotypes (15.6 vs. 5.0%, logistic regression, *Wald* $\chi^2[1]=9.10$, $P=0.003$), and was independent of their IG profiles ($P>0.6$). Moreover, the probability of pupal survival to adulthood was four times higher on Finnish than on Dutch genotypes (56.0 vs. 12.9%, logistic regression, *Wald* $\chi^2[1]=17.39$, $P<0.001$), also independent of their IG profiles ($P>0.3$). The greater survival to adulthood by Finnish pupae was not mediated by pupal mass, which did not differ between individuals raised on Finnish vs. Dutch genotypes (148 vs. 140 mg, $F[1,20]=0.76$, $P=0.39$).

Effects of Iridoid Glycosides on the Generalist and Specialist Parasitoids

Generalists Overall, associations between plant IGs and development time of the generalist parasitoids were not concordant with those observed for their hosts. Larvae of the parasitoid *C. marginiventris* developed faster on *C. chalcites* (Fig. 2c), and their pupae developed faster on *S. exigua* (Fig. 2d) when these herbivores had been feeding on genotypes with high IG concentrations (Table 1). An increase in IG concentration from 1% to 6% of leaf dry weight was accompanied by advancement in larval and pupal development by 19% on these hosts, (1.9 and 1.1 days, respectively). Effects on longevity were more concordant between hosts and parasitoids. In accordance with its host *S. exigua*, adult longevity of *C. marginiventris* decreased with higher IG concentrations in the diet of this host (Fig. 3b; $r=-0.580$, $P=0.04$). An increase in IG concentration from 1% to 6% of leaf dry weight reduced longevity by 51% (7.2 days). Adult longevity of the other generalist parasitoid, *H. didymator,* also on average decreased

with increasing IG concentration in the diet of its host *S. exigua* (Fig. 3C), but too few parasitoids on this host survived until adulthood to allow meaningful tests of significance. None of the other developmental parameters of *H. didymator* were significantly affected by IG concentration (Table 1, Fig. 2e,f).

Some of the developmental parameters were not associated with the total concentration of IGs in the plant genotypes on which hosts were raised but only with the specific compounds aucubin or catalpol. Survival of *C. marginiventris* pupae to adulthood decreased with the concentration of catalpol in the plant genotypes fed to its host on *S. exigua* (logistic regression, *Wald* $\chi^2[1]$=5.60, P=0.02). An increase in catalpol from 0.3% to 1.8% of leaf dry weight, the range encompassed by the genotypes, more than doubled the mortality of *C. marginiventris* pupae, from 14.5% to 31.8%. No such effects were observed for aucubin (*Wald* $\chi^2[1]$=0.41, P=0.52). Pupal mass of *H. didymator* decreased with increasing ratio of catalpol-to-total IG in the plant genotypes fed to its host *C. chalcites* (r=−0.827, P=0.002) on *C. chalcites*. The reduction in pupal mass of this parasitoid observed on the other host, *S. exigua*, was not statistically significant (r=−0.766, P=0.076).

Specialist On 15 of the 26 plant genotypes that were placed into natural host populations in the field, at least one *M. cinxia* caterpillar was parasitized by *C. melitaearum*. In total, 3.2% of the caterpillars were parasitized, with a mean secondary brood size of 2.1 parasitoids per parasitized caterpillar. This is a normal rate of parasitism of pre-diapause *M. cinxia* larvae by *C. melitaearum* in Åland (Lei et al., 1997). The parasitism rate was not significantly affected by the level of IGs in the plant genotypes that hosts had been feeding on before diapause. The parasitism rate on average decreased with the concentration of catalpol in the genotypes on which *M. cinxia* had been raised (Fig. 4b), but this pattern was not statistically significant (logistic regression, *Wald* $\chi^2[1]$=1.70, P=0.19). Accordingly, the parasitism rate was on average lower on Finnish (1.05%) genotypes (that had relatively high catalpol concentrations) than on Dutch genotypes (3.93%), but this difference was also not statistically significant (logistic regression, *Wald* $\chi^2[1]$=2.81, P=0.09). None of the post-diapause developmental parameters of *C. melitaearum* were significantly associated with post-diapause levels of IGs in the herbivore's diet.

Discussion

Our study supports findings from earlier studies showing that the development of generalist and specialist insect herbivores and their parasitoids is often strongly associated with intra- or inter-specific variation in concentrations of secondary plant compounds (e.g., Barbosa, 1988; Barbosa et al., 1991; Adler et al., 1995; Harvey et al., 2005, 2007a; reviewed by Ode, 2006). Our results show that the strength and direction of such associations may not only differ between generalists and specialists but also between trophic levels: responses in development time to plant allelochemical variation differed between herbivores and their associated parasitoids.

Herbivores Larval development of the generalist herbivore *C. chalcites* and pupal development of the generalist herbivore *S. exigua* were significantly delayed on plant genotypes with high levels of IGs. This increases their development time, potentially decreasing the intrinsic rate of increase at the population level. It also may increase their window of susceptibility to natural enemies, as in the 'slow-growth high-mortality' hypothesis (Clancy and Price, 1987), which states that herbivores are potentially more vulnerable to larval and pupal parasitoids, pathogens, and predators when they feed on host plants with high levels of allelochemicals that extend their development time. Our results confirm those of earlier studies (Biere et al., 2004) showing that development time of *S. exigua* is prolonged on *P. lanceolata* genotypes selected for high leaf IG concentrations, but the results contrast with studies of Harvey et al. (2005) in which *S. exigua* only suffered reduced pupal weight on high-IG genotypes. Apparently, the stage and specific parameters at which negative associations between host IG and development are expressed can vary even within a single system. In the current experiment, feeding on high-IG genotypes was not associated with reduced pupal or adult weight of the generalist herbivores, traits, which are generally correlated with fecundity (Leather, 1988; Klingenberg and Spence, 1997; Saastamoinen, 2007). Only longevity of adult *S. exigua*, another trait that may be associated with fecundity, was reduced on high-IG plant genotypes. Interestingly, both *C. chalcites* and *S. exigua* showed large variation in pupal and adult mass on different plant genotypes, which was unrelated to differences in IGs among these genotypes. Apparently, traits other than IGs varied among these genotypes, and played a role in determining pupal and adult mass of the generalist insect herbivores.

With respect to associations between IGs and development time, the results for the specialist herbivore *M. cinxia* contrast with those for the two generalist herbivores. Whereas development time of the generalists was delayed on plant genotypes with high total leaf IG concentrations, that of *M. cinxia* was not. Development time of this specialist only responded to variation in the profile, not the total concentration, of leaf IGs; larvae developed more quickly on genotypes with high catalpol-to-total IG ratios.

Contrasts between specialist and generalist herbivores in their development time responses to the total leaf IG concentration in their hosts have been observed previously. Harvey et al. (2005) found that *M. cinxia* caterpillars developed faster on high-IG than on low-IG genotypes (see also Saastamoinen et al., 2007), whereas development time of the generalist *S. exigua* was unaffected by IG variation among genotypes. It is unclear why in our study the development time of this generalist was longer on high-IG genotypes than in the study of Harvey et al. (2005). One reason might be that we used a wider range of IG variation among genotype means (0.8–6.4% dw) than was encompassed by the two lines used by Harvey et al. (2005) (0.6 vs. 3.0% dw). However, what these studies have in common is the observation that on high-IG plants the development of the specialist is either less delayed (our study), or even more advanced (Harvey et al., 2005) compared to that of generalist herbivores. Similar observations have been made for other allelochemicals. For instance, pyrrolizidine alkaloids (PAs) commonly impact generalist herbivores, even though the strength of their effect may differ strongly among generalist species (Macel et al., 2005), but not specialists (Macel et al., 2002). These results support the general notion that specialized mono- or oligophagous herbivores are often better able to cope with allelochemicals in the host on which they are specialized than polyphagous herbivores, at least in terms of their development rate.

Our results correspond well with results from artificial diet studies with IGs (e.g., Bowers and Puttick, 1988; Puttick and Bowers, 1988; Mallampalli et al., 1996). These studies have shown that addition of aucubin and catalpol to artificial diet strongly reduces larval growth rates, at least during the early stages of growth, of the generalist caterpillars *Lymantria dispar* and *Spodoptera eridania*, but can increase larval growth rate of the specialist *Junonia coenia* (Bowers and Puttick, 1988), even though this specialist generally experiences slightly lower feeding efficiency on high- compared to low-IG artificial diets (Camara, 1997a; Smilanich et al., 2009).

Artificial diet studies provide more direct evidence for causal links between IGs and insect development than our correlative studies based on plant genotypes that differ in IGs, because traits that covary with IGs in the plant might affect insect performance as well. We tried to minimize such associations by using a core set of genotypes derived from artificial selection lines that had been subjected to four generations of selection for low and high leaf IG concentrations (Marak et al., 2000). During selection, pre-existing associations are generally broken up, unless they are caused by pleiotropy or very close linkage. Indeed, analysis of a subset of plants from the 13 genotypes that were used in all experiments of the current study revealed that among-genotype correlations between concentrations of leaf IGs and concentrations of leaf nutrients, which are also important determinants of development of insect herbivores, were non-significant (Pearson correlation coefficients between IGs and N: r=+0.31, P=0.31; P: r=+0.26, P=0.39; K: r=+0.34, P=0.26, J. H. Reudler, unpublished results). This suggests that our results probably have not been confounded with effects of co-varying levels of primary metabolites.

The large differences in post-diapause development of the specialist herbivore *M. cinxia* on the Finnish and Dutch *P. lanceolata* genotypes however, does illustrate the importance of other plant traits, in addition to IGs, in governing the interactions between *P. lanceolata* and its insect herbivores. Developmental success of *M. cinxia* was much higher on their local, Finnish, genotypes than on the Dutch genotypes: they developed faster and showed higher larval and pupal survival on the local genotypes. Finnish genotypes had consistently higher levels of catalpol than Dutch genotypes, a difference that was reflected in higher ratios of catalpol to the total amount of IGs. To our surprise, only the faster development of post-diapause *M. cinxia* larvae on Finnish than on Dutch genotypes could be (partly) explained by the differences in their IG profiles. Differences in IG profiles between Finnish and Dutch genotypes could not explain the large differences in post-diapause larval and pupal survival of *M. cinxia* on these genotypes. This suggests that *M. cinxia* has adapted to feeding on its local hosts at least partly in response to attributes other than their IG chemistry. Adaptation of insect herbivores to their local host plants is common, though not ubiquitous (Kaltz and Shykoff, 1998; Ballabeni et al., 2003; Greischar and Koskella, 2007), and the traits that underlie local adaptation may involve many aspects other than those involved in allelochemical-based interactions (e.g., Zovi et al., 2008).

Parasitoids The performance of the two generalist parasitoids on the generalist herbivores was associated strongly with the leaf IG concentrations in the plant genotypes on which these hosts were raised. In contrast to earlier studies (Mallampalli et al., 1996; Harvey et al., 2005), the magnitude and even direction of the correlations between IGs and performance of the parasitoids did not follow that of the hosts. Notably, whereas both of the generalist herbivores developed slowly on plant genotypes with high IG concentrations, the parasitoid *C. marginiventris* actually developed faster on both host species when they were reared on high-IG genotypes. The faster development of *C. marginiventris* on hosts that suffered from feeding on high-IG plants is surprising, since negative effects of the ingestion of toxic allelochemicals on the performance and quality of herbivores often have been observed to vertically transfer to higher trophic levels (Campbell and Duffey, 1979; Barbosa et al.,

1986, 1991; Harvey et al., 2007a; Lampert et al., 2008), especially in interactions where hosts sequester toxic allelochemicals (part of the "nasty host" hypothesis; Gauld et al., 1992, Lampert et al., 2010). One possible explanation for the faster growth of *C. marginiventris* on these hosts is that higher levels of allelochemicals in the host diet weaken the host immune system, as observed for *J. coenia* feeding on high-IG artificial diets (Smilanich et al., 2009), thus allowing the developing parasitoids to reallocate metabolic energy from immunosuppression to development (Kraaijeveld and Godfray, 1997; Ojala et al., 2005), the "immunocompromized host" effect. This hypothesis remains to be confirmed for the species studied here. Associations between adult longevity and host IGs were more concordant between herbivores and parasitoids. Longevity of adult *S. exigua* was significantly reduced on high-IG genotypes and so was that of *C. marginiventris,* when raised on this host. Similarly, longevity of both adult *C. chalcites* and its parasitoids was not affected by leaf IG concentration in the host's diet.

The effect of IGs in the diet of the generalist herbivores on the performance of their parasitoids strongly differed between the two parasitoid species. A high concentration of IGs in the diet of both host species resulted in faster development of *C. marginiventris,* despite slower development of its hosts, but did not affect the development of *H. didymator.* This illustrates that even when we consider interactions with a specific group of parasitoids, such as generalist endo-koinobionts, effects of IGs in the diet of an herbivore on interactions with its parasitoids cannot be generalized. Part of the difference between parasitoid species may be due to their different feeding modes. *Cotesia marginiventris* is a haemolymph feeder whereas *H. didymator* feeds on tissue. Haemolymph feeders may be less constrained by host growth rate than tissue feeders that have to consume their host before being able to emerge (Harvey et al., 2000; Harvey and Strand, 2002). Previous studies similarly found that the related tissue feeder *Hyposoter horticola* more closely followed developmental responses of the host *M. cinxia* to IG variation than the haemolymph feeder *C. melitaearum* (Harvey et al., 2005).

In contrast to the generalist parasitoids, development of the specialist parasitoid *C. melitaearum* did not vary with the IG levels in the plant genotypes on which its host *M. cinxia* was reared, in agreement with the results of Harvey et al. (2005). Nieminen et al. (2003) found that in natural populations, the rate of parasitism of *M. cinxia* by *C. melitaearum* decreased with the concentration of catalpol in the *P. lanceolata.* Although we found the same trend in our experiment, the decrease was not statistically significant, perhaps due to small sample size. Effects of host plant IGs on parasitism rates could result from two different processes. First, adult parasitoids may avoid ovipositing into host larvae feeding on high-IG plants. Second, development of immature parasitoids may be less successful on hosts feeding on high IG plants. For instance, whereas field parasitism of the Catalpa sphinx moth (*Ceratomia catalpa*), a species that sequesters IGs (Bowers, 2003), by the parasitoid *Cotesia congregata* appears to be independent of caterpillar IG concentrations, survival of this parasitoid is slightly lower on high-IG caterpillars (Lampert et al., 2010). *Melitaea cinxia* also is known to sequester IGs (Suomi et al. 2001, 2003) and development of *C. melitaearum* larvae on high-IG caterpillars may be less successful if the negative effects due to the vertical transfer of these toxic allelochemicals (the "nasty host" effects) more than outweigh any positive effects due to decreased immunocompetence of the host (the "immunocompromized host" effect). Interestingly, in contrast to *J. coenia*, that is immunocompromized when feeding on high-IG diets (Smilanich et al., 2009), Laurentz (2010) found no evidence for a decreased immune function in *M. cinxia*. In fact, *M. cinxia* showed higher levels of encapsulation of artificial implants when reared on plants with high levels of catalpol.

In summary, we found that development time of the specialist herbivore was less negatively impacted by high-IG genotypes than that of both of the generalists, supporting the idea that specialist herbivores are better adapted to allelochemicals in their hosts than are generalist herbivores. We also found that generalist parasitoids developed faster on hosts feeding on high-IG genotypes whereas the specialist parasitoid was not strongly impacted by the IG concentration in the diet of its host, which could reflect differences in the effects of IGs on immunocompetence on the different hosts that they parasitized. Our study, therefore, shows that associations between IG concentrations in *P. lanceolata* and development of its associated insect herbivores and parasitoids are species-specific.

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Elevated Carbon Dioxide and/or Ozone Concentrations Induce Hormonal Changes in *Pinus tabulaeformis*

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Abstract We investigated endogenous plant hormones and needle growth in *Pinus tabulaeformis* plants grown in open-top chambers and exposed to ambient or elevated concentrations of carbon dioxide (CO_2) and/or ozone (O_3). Exposure to elevated CO_2 for 100 days significantly increased the change in fresh needle weight, indole-3-acetic acid (IAA), isopentenyl-adenosine (iPA), and dihydrozeatin riboside (DHZR) content. Abscisic acid (ABA) content decreased, and no effect was observed on zeatin riboside (ZR) content or changes in needle dry weight. The ratios of IAA/ABA and total cytokinins (CKs)/ABA $(ZR + DHZR + iPA/ABA)$ were increased. Elevated O_3 significantly decreased IAA and ZR, and decreased the ratios of IAA/ABA and CKs/ABA. Ozone treatment increased ABA content but did not change iPA or DHZR content or change fresh or dry needle weights. The combination treatment significantly increased ABA content and the IAA/ABA ratio but decreased the total CKs/ABA ratio and had no effect on CKs or IAA content or change in fresh and dry needle weights. The results indicate that elevated CO_2 ameliorated the effects of elevated O_3 on tree growth.

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Key Words Ozone · Elevated CO_2 · Elevated O_3 · Endogenous plant hormones · Needle growth

Introduction

Levels of the greenhouse gases carbon dioxide (CO_2) and ozone (O_3) in the atmosphere are increasing because of human activities. Atmospheric CO_2 concentration has increased from 280 ppm in pre-industrial times to 379 ppm in 2010 and is predicted to at least double by the end of this century (IPCC, 2007). Anthropogenic emissions of O_3 precursors have increased significantly in China, in contrast to the small changes in North America and significant decreases in Europe (Streets and Waldhoff, 2000). Such changes in the atmospheric environment are expected to affect the growth and development of plants (Darbah et al., 2008; Riikonen et al., 2008). Plant hormones mediate plant responses to environment and plant development, and they communicate environmental conditions to the entire plant. Different parts of the plant are programmed to respond to hormonal levels by changing gene expression, thus increasing the chances that the individual will survive the changed environment (Winwood et al., 2007).

Leaf growth is often enhanced following exposure to elevated CO_2 (Taylor et al., 2003; Riikonen et al., 2004; reviewed in Lindroth, 2010). Li et al. (2002) and Teng et al. (2006) demonstrated that indole-3-acetic acid (IAA) levels increase under elevated CO_2. It is well known that IAA is important for cell division, elongation, and apical dominance (del Pozo et al., 2005; Mockaitis and Estelle, 2008) and that it promotes cell wall extensibility by inducing expression of expansin genes (Caderas et al., 2000; Greenwood et al., 2006). In *Populus* spp., the increase in leaf area expansion rate by elevated CO_2 was associated with increased cell wall

extensibility and increased activity of the cell wall-loosening enzyme (Ferris et al., 2001).

Natural cytokinins (CKs) induce cell division and shoot formation (Miller et al., 1955; Skoog and Miller, 1957), increase sink strength (Richmond and Lang, 1969), and delay senescence (Gan and Amasino, 1995). Dihydrozeatin (DHZ), isopentenyladenine (iPA) and zeatin (Z) are the predominant CKs found in higher plants. The free bases and their ribosides [dihydrozeatin riboside (DHZR), isopentenyladenine riboside (iPAR), and zeatin riboside (ZR)] are thought to be the biologically active forms of the CKs. The auxin-CKs hypothesis predicts that CKs, together with auxin, play an essential role in plant morphogenesis, influencing the formation of roots and shoots and their relative growth rates (Skoog and Miller, 1957). Some evidence indicates that accelerated growth and development observed under elevated CO_2 is accompanied by a corresponding increase in CKs concentrations (Li et al., 2002; Teng et al., 2006), while the slowed growth and development observed under elevated O_3 is accompanied by a decrease in CKs concentrations (Winwood et al., 2007). The role of CKs as retardants of leaf senescence under stress conditions has been documented both when exogenously supplied (Wingler et al., 1998) and in transgenic plants with elevated endogenous CKs levels (Gan and Amasino, 1995; Jordi et al., 2000).

Abscisic acid (ABA) regulates diverse plant processes, including growth and development under non-stress conditions, and plays a pivotal role in abiotic stress tolerance (Chinnusamy et al., 2008). Recently, evidence has been found for a role in community succession (Zhao et al., 2011). ABA is generally classified as a hormone with growth-inhibiting activity, but Cheng et al. (2002) reported a growth-promoting role for ABA in controlling fertility, vegetative growth, and organ and body size in the absence of stress conditions. These contrasting roles could be explained if ABA activates growth at low concentrations but inhibits growth at high concentrations (Cheng et al., 2002). ABA levels can change in response to environmental conditions. For example, ABA levels increase in response to elevated O_3 in *Pinus cembra* needles (Bianco and Dalstein, 1999) and decrease in response to elevated CO_2 in *Mokara yellow* and *Arabidopsis thaliana* leaves (Li et al., 2002; Teng et al., 2006).

In this study, hormone levels were determined in the needles of *P. tabulaeformis* grown under elevated CO_2, O_3, or CO_2 and O_3. We hypothesized that elevated CO_2 might increase the growth-promoting hormones and decrease the growth-inhibiting hormones in the needles of *P. tabulaeformis*, while elevated O_3 might have opposite effects. Furthermore, we hypothesized that when both CO_2 and O_3 were elevated, the CO_2 might ameliorate the negative effects of elevated O_3 on growth.

Methods and Materials

Experimental Site The experiment was conducted at the Shenyang Arboretum at the Chinese Academy of Science, located in the populated area of Shenyang, China (41°46′N, 123°26′E). The average annual temperature is 7.4°C, while the average temperatures in January and July are −12.6°C and 27.5°C, respectively. The highest temperature was 38.3°C, the lowest temperature was −32.3°C, and the average annual precipitation is 755.4 mm in this area.

Plant Material Four–yr-old Chinese pine (*Pinus tabulaeformis*) saplings (about 0.7–1.0 m high), all from a single maternal plant, were purchased from a local nursery. All saplings were planted in open-top chambers (OTCs), 20 saplings per chamber. The soil was loamy, and supplemental fertilizer was provided to ensure that saplings were not nutrient-limited. Rainfall was occasionally supplemented to minimize water stress.

Experimental Design The factorial design of 12 OTCs (diam 4 m, height 3 m, with 4 m distance between neighboring OTCs) containing 3 OTCs with ambient air, 3 with elevated CO_2, 3 with elevated O_3, and 3 with the combination of elevated CO_2 and O_3 treatment was randomized in four blocks. Pure CO_2 was injected into the chambers from cylinders 24 h per day. Ozone was produced by electrode discharge (O_3 generator, model GP-5J, Jiangsu Xinghua Environmental Protection Science and Technology Limited Company, Taixing, China) from bottled oxygen and added to the OTCs 9 h per day (08:00–17:00). The CO_2 and O_3 concentrations in the chambers were monitored with a CO_2 infrared transducer (eSENSE-D; SenseAir, Delsbo, Sweden) and an O_3 transducer (S-900; Aeroqual, Auckland, New Zealand). Mean temperature was approximately 25°C (max. 35.7°C; min. 11.4°C), and mean relative air humidity was 50.2% in the OTCs.

Experimental Treatment For 100 d from 17 June to 27 September 2007, saplings in the OTCs were exposed to control (ambient CO_2, 350 μmol mol^{-1} and ambient O_3, 55 nmol mol^{-1}, designated AA), elevated CO_2 (CO_2, 700 μmol mol^{-1} and ambient O_3, designated EC), elevated O_3 (ambient CO_2 and O_3, 80 nmol mol^{-1}, designated EO), and the combination treatment (CO_2 and O_3, 700 μmol mol^{-1} and 80 nmol mol^{-1}, respectively, designated EC+EO). For each OTC, one fascicle (two needles) was randomly taken from the stem apex of every sapling on June 16 (before fumigation) and on September 27 (after 100 d of fumigation). Needles collected on day zero and needles collected on d 100 of fumigation were each pooled to obtain two samples per chamber for analysis of fresh and dry weight (dried at 70°C for 48 h). Samples (1.0 g) were collected from each OTC

before fumigation and on days 20, 40, 60, 80, and 100 of fumigation washed in distilled water, frozen in liquid nitrogen, and stored at −70°C for subsequent hormone analyses.

Hormone Determination The frozen samples (1.0 g) were powdered in liquid nitrogen and extracted at 4°C for 16 h in the dark with 80% (*v*/*v*) cold methanol containing 1 mM butylated hydroxytoluene as an antioxidant, and centrifuged at 16,000 × *g* for 15 min at 4°C. The residues were re-suspended in the same ice-cold extraction solution at 4°C for 1 h and recentrifuged. The supernatants were combined, passed through Chromosep C18 columns (C18 Sep-Park Cartridge, Waters, Millford, MA, USA), dried under N_2, and dissolved in 2 ml phosphate-buffered saline (PBS) containing 0.1% (*v*/*v*) Tween 20 and 0.1% (*w*/*v*) gelatin (pH 7.5) for analysis by an enzyme-linked immunosorbent assay (ELISA) following methods described in previous publications (Yang et al., 2001; Teng et al., 2006). The mouse monoclonal antibodies against the hormones, and Igg-linked horseradish peroxidase, were produced at the Phytohormones Research Institute (China Agricultural University, Beijing, China). Each antibody had high immunoreactivity and showed low cross reactivity with other hormones.

Statistical Analysis All hormone measurements were performed on extracts of plant tissue from each chamber, and these independent measurements were averaged to obtain a chamber mean for each sample date. Chambers corresponding to AA, EC, EO, and EC+EO were considered statistical replicates (N=3). Two-way ANOVA was carried out using the SPSS statistical software package (SPSS Inc., Chicago, IL, USA) for all sets of data at each sampling day. The values presented are the means of all measurements, and comparisons of means were determined through LSD test.

Results

Changes in Growth under Elevated CO_2 and/or O_3 In this experiment, the increase in fresh mass (per needle) during the exposure period from day 1 to day 100 was larger under elevated CO_2 compared to AA control (P<0.05), but the increase in dry mass showed no significant change. Elevated O_3 and the combination treatment had no significant effect on the increases in fresh and dry masses (Table 1).

Changes in Endogenous CKs under Elevated CO_2 and/or O_3 Elevated CO_2 or the combination treatment did not induce any significant change in ZR content compared with the AA control, but elevated O_3 decreased ZR content at days 20 and 80 (P<0.05) (Fig. 1a).

Table 1 Effects of elevated CO_2 (EC), elevated O_3 (EO) and elevated CO_2+O_3 (EC+EO) on increase in needle mass of *Pinus tabulaeformis* from June 16 (before fumigation) to September 27 (after 100 days of fumigation). Different letters between treatments indicate significant difference at P<0.05

Treatment	Increase in fresh weight per needle (g)	Increase in dry weight per needle (g)
AA	0.221±0.0114a	0.107±0.0135ab
EC	0.277±0.0138b	0.115±0.0116b
EO	0.194±0.0131a	0.086±0.0125a
EC+EO	0.226±0.0144a	0.094±0.0128ab

From day 20 to day 80, elevated CO_2 increased DHZR content (P<0.05 at day 20, 60, and P<0.01 at day 40, 80), while from day 40 to day 80, O_3 fumigation increased DHZR content in the needles compared with the AA control (P<0.05 at day 60 and P<0.01 at day 40, 80). DHZR content during the combination treatment was not significantly different from the AA control (Fig. 1b).

Under elevated CO_2, iPA content was higher at day 20 (P<0.05) and 80 (P<0.01), while elevated O_3 decreased iPA content only on day 100 (P<0.05). Compared with the AA control, the combination treatment induced no significant change in the iPA content (Fig. 1c).

Changes in Endogenous IAA and ABA under Elevated CO_2 and/or O_3 IAA content was higher under elevated CO_2 than in the AA control (P<0.01). No significant change was observed under elevated O_3 (except at day 80) or under the combination treatment throughout the experiment (Fig. 1d). Elevated CO_2 decreased ABA content (P<0.05 at days 20 and 80), while elevated O_3 and the combination treatment increased ABA content (P<0.01 at days 20 and 60) (Fig. 1e).

Changes in Hormone Ratios under Elevated CO_2 and/or O_3 Elevated CO_2 significantly increased the ratios of IAA/ABA and total CKs/ABA $[(ZR + DHZR + iPA)/ABA]$ relative to the AA control. The ratios were lower under elevated O_3 (Fig. 2a, b). The combination treatment significantly increased the IAA/ABA ratio and significantly decreased the CKs/ABA ratio, but the changes were not as large as those obtained with the single treatments.

Discussion

Hormones act as signals that can stimulate or inhibit growth and regulate developmental programs in plants (Fosket, 1994). We showed that elevated CO_2 and elevated O_3 have opposite effects on plant growth. Generally, elevated CO_2

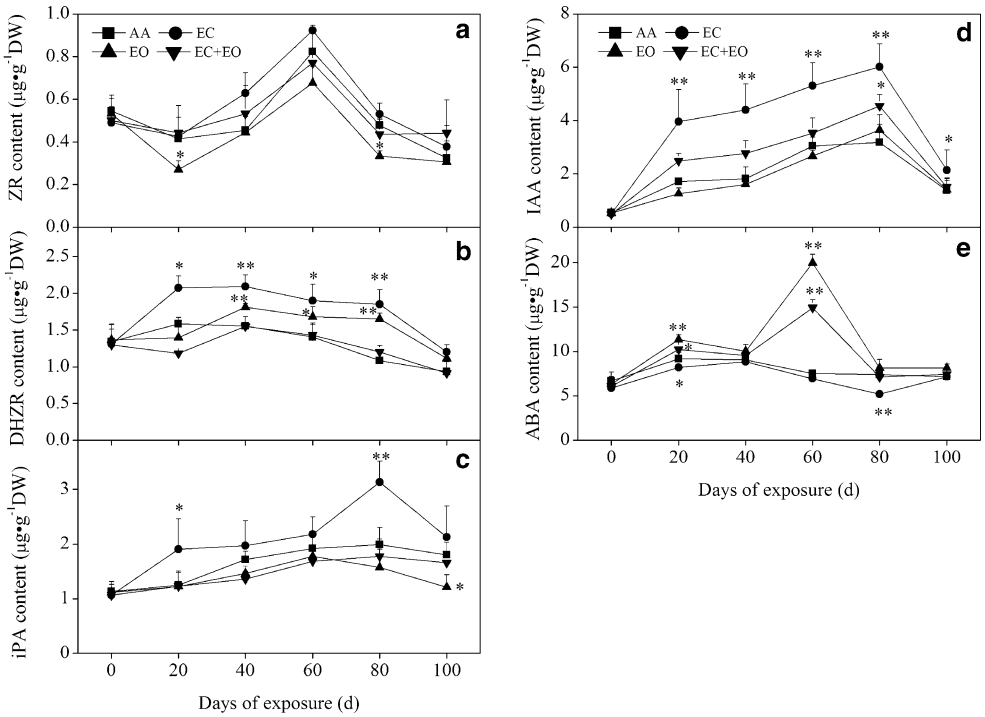


Fig. 1 Effects of ambient (*AA*), elevated CO_2 (*EC*), elevated O_3 (*EO*) and elevated CO_2+O_3 (*EC*+*EO*) on ZR (**a**), DHZR (**b**), iPA (**c**), IAA (**d**) and ABA (**e**) in *Pinus tabulaeformis* needles. Data are means of three independent open-top chambers (±SD). Significant differences from the AA control are indicated with asterisks: * $P<0.05$; ** $P<0.01$

increased the accumulation of growth-promoting hormones (IAA, CKs), and elevated O_3 increased the accumulation of the growth-inhibiting hormone ABA. The combination of elevated CO_2 and elevated O_3 had a little effect on hormone levels.

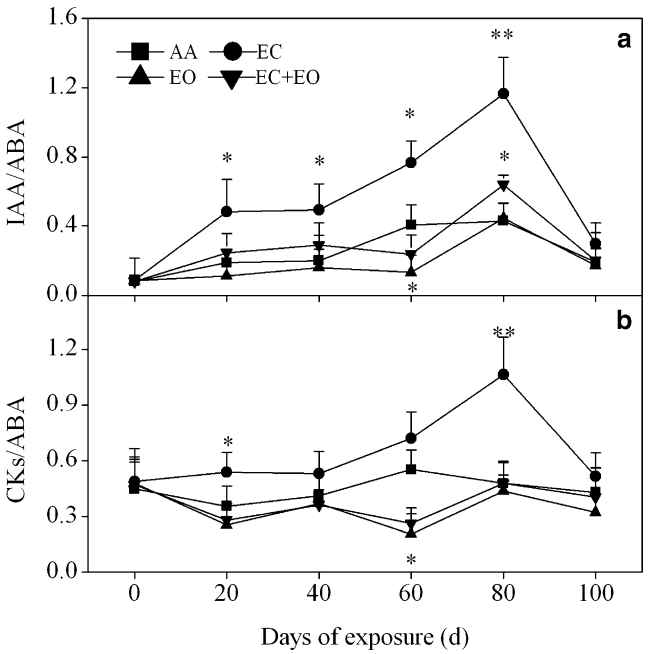


Fig. 2 Effects of ambient (*AA*), elevated CO_2 (*EC*), elevated O_3 (*EO*) and elevated CO_2+O_3 (*EC*+*EO*) on hormonal ratios IAA/ABA (**a**) and CKs/ABA (**b**) in *Pinus tabulaeformis* needles. Data are means of three independent open-top chambers (±SD). Significant differences from the AA control are indicated with asterisks: * $P<0.05$; ** $P<0.01$

Many researchers have reported that elevated CO_2 accelerates the net assimilation rate and enhances plant growth (King et al., 2005; Zheng et al., 2010). In contrast to the beneficial effects of elevated CO_2, O_3 is generally detrimental to plant growth and metabolism (Darbah et al., 2008; Lindroth, 2010; Noormets et al., 2010; Pinto et al., 2010). In our experiment, the change in fresh weight (per needle) was significantly increased by elevated CO_2. The change in fresh and dry weight (per needle) was slightly reduced by elevated O_3 compared with control although the change did not reach statistical significance. Cell division and expansion are controlled by a complex network of factors, including phytohormones that respond to environmental cues (Nishimura et al., 2004; Tsukaya, 2006). Taylor et al. (2003) suggested that the effects of elevated CO_2 on the cell cycle are likely to involve an interaction between plant hormones and carbon status. Accelerated *Arabidopsis thaliana* growth and development under elevated CO_2 could in part be attributed to increased foliar concentrations of plant hormones (Teng et al., 2006). We also observed that elevated CO_2 increased the levels of growth-promoting hormones (IAA, ZR, DHZR, and iPA). Therefore, we propose that elevated CO_2 levels may enhance plant growth by increasing hormone concentrations. Furthermore, we found that exposure to elevated O_3 levels decreased IAA content, which in turn may have inhibited plant growth. Somewhat lower needle growth rate accompanied lower concentrations of some hormones (IAA, ZR, and iPA) under elevated O_3.

The data presented here demonstrate that elevated CO_2 was associated with an increase in iPA and DHZR content,

which are likely responsible for the increase in growth and photosynthesis, as reported by other groups (Ookawa et al., 2004). Exogenous CKs application has been shown to attenuate the deleterious effects of O_3, perhaps because CKs may act as radical scavengers, protecting plant tissue from damage by O_3 (Pauls and Thompson, 1982). Our results show that under elevated O_3 there was an increase in DHZR content but no changes in iPA and ZR content. We speculate that the increase in DHZR content might enhance antioxidant capability to alleviate the oxidative stress of elevated O_3.

ABA traditionally has been considered an inhibitor of growth (Zhang and Davies, 1990) while other roles of ABA are less well defined. In the present study, we found that ABA content decreased under elevated CO_2 and increased under elevated O_3, and this change may be related to the corresponding increase or decrease in growth of *P. tabulaeformis*.

Plant growth and development is not governed by a single hormone but by the interaction of several. For example, Bettini et al. (2010) reported that a lower IAA/ABA ratio in the rolC-transgenic tomato can lead to reduced vegetative growth. A relationship between shoot development and the ratio of these two hormones was observed in lemon scions, in which a vigorous rootstock had a higher IAA/ABA ratio in the new shoots (Noda et al., 2000). We noted a relationship between increased vegetative growth and elevated IAA/ABA ratio and CKs/ABA ratio under elevated CO_2. In contrast, the IAA/ABA ratio is lower when plants are stressed (Zhang et al., 2008), consistent with our observations under elevated O_3 levels.

Little is known about the effect of simultaneously elevated CO_2 and O_3 on hormone levels. Our results indicate that EC+EO increased both the growth-promoting hormone IAA and the growth-inhibiting hormone ABA. It is well known that phytohormone metabolism is dependent on the supply of carbohydrates (Taiz and Zeiger, 2002). Under the combination of elevated CO_2 and O_3, more substrate and energy are required to prevent or repair injury (Paoletti and Grulke, 2005). Indeed, the level of the CKs that can enhance cell growth and development showed no change, but ABA, which can enhance plant resistance, was increased. The increase in IAA content induced by EC+EO may be related to increased CO_2 amelioration, but the mechanism behind these responses is still unclear.

In summary, our results show that elevating atmospheric CO_2 concentration alone enhances the growth of *P. tabulaeformis*. There is no growth enhancement when O_3 concentration is elevated, and there are reductions in IAA and CKs content and an increase in ABA content. There was no significant effect of the combined treatment with elevated CO_2 and elevated O_3 on hormone levels, probably because the elevated O_3 reduced the growth-promoting effects of elevated CO_2 or because elevated CO_2 counteracted the negative impact of elevated O_3.

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Abscisic Acid in Soil Facilitates Community Succession in Three Forests in China

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Abstract Plants release secondary metabolites into the soil that change the chemical environment around them. Exogenous abscisic acid (ABA) is an important allelochemical whose role in successional trajectories has not been examined. We hypothesized that ABA can accumulate in the soil through successional processes and have an influence on forest dynamics. To this end, we investigated the distribution of ABA in forest communities from early to late successional stages and the response of dominant species to the gradient of ABA concentrations in three types of forests from northern to southern China. Concentrations of ABA in the soils of three forest types increased from early to late successional stages. Pioneer species' litters had the lowest ABA content, and their seed germination and seedling early growth were the most sensitive to the inhibitory effect of ABA. Mid- and late-successional species had a much higher ABA content in fallen leaves than pioneer species, and their seed germination and seedling early growth were inhibited by higher concentrations of ABA than pioneers. Late-successional species showed little response to the highest ABA concentration, possibly due to their large seed size. The results suggest that ABA accumulates in the soil as community succession proceeds. Sensitivity to ABA in the early stages, associated with other characteristics, may result in pioneer species losing their advantage in competition with late-successional species in an increasingly high ABA concentration environment, and being replaced by ABA-tolerant, late-successional species.

Key Words ABA concentration · Acceleration · Allelopathy · Bioassay · Community succession · ELISA quantification · Leaf litter · Seed germination · Seed mass · Seedling growth

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Introduction

Ecological succession is the result of interactions between vegetation and environmental factors and it is determined by multiple processes. Previous studies of community succession have identified numerous mechanisms that govern vegetation dynamics. These include physical factors [e.g., climatic (Kröpelin et al., 2008)], changes in soil moisture and nutrition (Gao et al., 2003), light interception (Facelli and Pickett, 1991b), and biological factors such as interactions between plants, life-history characteristics, seed predation (Facelli, 1994), and herbivory (Wilby and Brown, 2001). Studies have identified the importance of allelopathy in forest renewal and species composition (Rietveld et al., 1983; Fernandez et al., 2006), and some authors contend

that the replacement of plant species during succession is partly due to allelopathic mechanisms (Rice, 1972; Reigosa et al., 1999). Phytotoxins, released by leaching, root exudates, volatilization, or litter decomposition, can affect seed germination (Ruprecht et al., 2008), seedling establishment (Bosy and Reader, 1995), and plant growth and distribution (Rice, 1972). Biochemical interactions differ in their strength and may differentially affect the competitive vigor of dominant and subordinate species (Ruprecht et al., 2008). Hence, to understand these interactions and the potential influence of dominant species on ecosystem processes, it is necessary to identify those plant species in a community with significant allelopathic effects on other species (Anderson et al., 1978). Similarly, it is important to reveal the differential susceptibility of community constituents to these toxins (Wardle et al., 1993).

We previously assessed the allelopathic potentials of a series of dominant woody species in different successional stages on other dominant species in a subtropical forest on Dinghu Mountain, South China. We found that the litter and soils beneath individuals of *Castanopsis chinensis*, one of the dominant tree species in the mid-successional stage, have substantial inhibitory effects on seed germination of pioneer species as well as on conspecifics, but little impact on late-successional species (Zhao et al., unpublished results). This suggests that the allelopathic effect of this tree may affect vegetation succession by accelerating early-successional species decay and late-successional species establishment. We isolated the allelopathic chemicals in *C. chinensis* and confirmed that abscisic acid (ABA) is the major growth inhibitor (Zhao et al., unpublished results).

The phytohormone ABA regulates various aspects of the physiological and developmental processes of plants, such as seed maturation, dormancy, and germination (Leung and Giraudat, 1998), as well as plants' responses to biotic and abiotic stresses (Finkelstein et al., 2002). Studies have identified ABA as an allelopahtic chemical (Buta and Spaulding, 1989; Tseng et al., 2003). ABA synthesized by plants can enter the environment through litter leachate, trunk sap, and radicle exudation, and is common in higher plants. Nanogram quantities of ABA were sufficient to cause inhibition on tall fescue seed germination and seedling early growth (Buta and Spaulding, 1989), indicating its importance as a plant growth regulator. Thus, this compound has the potential to have an influence on vegetation composition and dynamics. The objectives of this work were: (i) to investigate the behavior of ABA in the process of community succession; (ii) to reveal the response of dominant species from different successional stages to environmental ABA; and (iii) to attempt to clarify the role of ABA in community succession.

Methods and Materials

Study Site We conducted our experiment in three forest types in three different national nature reserves in China (map in Supplementary Fig. S1): (i) Boreal mixed forest on Changbai Mountain (41°31′–42°28′N, 127°09′–128°55′E) in the southeast of the Jilin province in northeastern China. Here, there were three plots arranged along an elevation gradient from 700–800 m above sea level. The study area is dominated by a cool temperate continental climate with four clearly defined seasons and a monsoon influence. The mean annual temperature ranges from 0.9°–3.9°C., and the mean annual precipitation ranges from 700–800 mm. The zonal soils are dark brown forest soils. The primary stage of succession at this site consists of *Betula platyphylla* + *Populus davidiana*, while the middle stage is mainly represented by *B. platyphylla* + *P. davidiana* + *Pinus koraiensis*. The climax stage typically consists of *P. koraiensis*. (ii) Evergreen broadleaf forest on Tianmu Mountain (30°18′–30°25′N, 119°24′–119°28′E) in the northwest of the Zhejiang province, the northern subtropical area of eastern China. At this site, there were four plots arranged along an elevation gradient from 300–750 m above sea level. This area is influenced by a monsoonal climate and a high altitude with steep slopes, heavy rainstorms in the spring and fall, and a large range of snowfall in winter. The mean annual temperature ranges from 8.8°–14.8°C., and the mean annual precipitation ranges from 1390–1870 mm. The zonal soils are comprised of red soils (below 600 m a.s.l.), yellow soils (600–1,200 m a.s.l.), and brown yellow soils (above 1,200 m a.s.l.). The pioneer vegetation is dominated by *Pinus massoniana*, the early mid-successional stage is conifer-broadleaf mixture forest characterized by *P. massoniana* + *Schima superba*, the later mid-successional stage is mixed broadleaf forest with *S. superba* + *Cyclobalanopsis gracilis* + *Castanopsis sclerophylla* + *Castanopsis eyrei*, and the mature forest consists of *C. gracilis* + *C. sclerophylla* + *C. eyrei*. (iii) Evergreen broadleaf forest on Dinghu Mountain (23°09′–23°12′N, 112°30′–112°34′E) in the central part of the Guangdong Province in southern China. Here, there were four plots arranged along an elevation gradient from 150–300 m above sea level. The region is characterized by a typical south subtropical monsoon climate, with annual average precipitation of 1927 mm, of which nearly 80% falls in the warm humid season (April–September) and 20% in the cool dry season (October–March). The mean annual temperature is 20.9°C and the relative humidity is 80%. The soils, with serious natural acidification and a pH of 4.1–4.9, are classified as hydration lateritic. There are four types of natural vegetation communities in this forest representing a sequence of successional stages from pioneer to climax. The sequence begins with a conifer (*P. massoniana*, the same species found

on Tianmu Mountain), followed by a conifer-broadleaf mixture (with *P. massoniana* + *Castanopsis chinensis* + *S. superba*), then an evergreen broadleaf forest (with *Ca. chinensis* + *S. superba* + *Castanopsis fissa*), and finally the climax stage is mesophytic forest (with *Ca. chinensis* + *C. fissa* + *Cryptocarya concinna* + *Crvptocarya chinensis*).

Plant Material We selected 12 dominant species from different successional stages in the 3 forests (Table 1). The seeds and leaf litter of the dominant species were collected randomly. There were 10 trees of each species selected for leaf litter collection. Seeds were stored at 4°C until use, and fallen leaves were oven-dried at 40°C for 48 h and then stored at room temperature until use. Ten surface soil samples (at 2–4 cm depth) of all successional phases were collected randomly by a handle soil collecting tool; macroscopic stones, litters, roots, and other organic debris were removed. Each soil sample was mixed and separated into two parts. One part was oven-dried at 105°C to a stable weight to measure moisture content, and the other part was stored at −20°C until used to measure ABA content (storage at this temperature minimizes any microbial activity and prevents ABA decomposition). The soils of Changbai Mountain were collected in May, and the soils of Tianmu Mountain and Dinghu Mountain were collected in April, the seasons of highest seed germination.

ABA Quantification ABA content in the leaf litter and soils was measured by using an immunoassay, according to the method described by Pelèse et al. (1989) and modified by Hocher et al. (1991). Samples (0.5 g dry weight) were crushed and extracted for 60 h at 4°C in darkness in 5 ml of aqueous methanol (80%) containing 40 mg l^{-1} BHT as antioxidant. Sample purification was achieved by filtration of the methanolic extracts through a 0.2 μm pore size filter connected with a C_{18} Sep-Pack cartridge (Waters, Milford, MA, USA), which was eluted with 80% aqueous methanol. Extracts then were reduced to dryness by nitrogen evaporation and re-dissolved with a phosphate buffer prior to ELISA quantification.

Bioassay Based on the soil ABA concentration data, and taking into account the lower biological activity of artificial ABA compared to natural ABA, we used a series of ABA concentrations to test the response of seed germination and seedling early growth of selected seeds. The 250 ml conical flasks were filled with 100 ml of 0.9% agar culture medium and sterilized at 120°C for 0.5 h, then filled with 100 μg/ml of ABA solution (Sigma Co. USA) to create cultures containing 0, 0.5, 1.0, 2.0, 4.0, or 8.0 μg/ml of ABA when the temperature of the culture medium fell to about 50°C. Thirty surface-sterilized and germination-accelerated seeds were sown on the culture medium and cultivated in a dark room at 25°C. Three replicates were processed. Seedlings were harvested 7–15 d after seeds germinated, and seed germination and total length of roots and shoots were calculated. Percent inhibition (I) of seed germination was calculated by using the following formula (Chung et al., 2001):

$$\mathrm{I}(\%) = [1-(\text{growth parameter in a treatment}/\text{growth parameter in control})] \times 100$$

Statistical Analysis Differences among means of successional series in ABA concentration and bioassays were analyzed with one-way ANOVA LSD tests. Differences among means of ABA content in litter in Changbai Mountain were analyzed using an independent-samples *t*-test (2-tailed). The relationships between seed mass and the inhibitory effect of ABA were analyzed by Pearson Correlation Coefficients. Statistical analyses were per-

Table 1 Selected dominant species in each of the three forests, their seed mass and successional status

Stations	Location	Species	Seed mass (mg)	Successional status
Changbai	42°24′N, 128°05′E	*Betula platyphylla*	0.1	early
		Pinus koraiensis	547.9	late
Tianmu	30°23′N, 119°26′E	*Pinus massoniana*	9.67	early
		Schima superba	4.7	middle
		Cyclobalanopsis gracilis	1089.5	middle
		Castanopsis sclerophylla	865.3	middle
		Castanopsis eyrei	813.1	later
Dinghu	23°11′N, 112°33′E	*Pinus massoniana*	9.34	early
		Castanopsis chinensis	838.9	middle
		Castanopsis fissa	1183.3	middle
		Cryptocarya concinna	823.1	late
		Crvptocarya chinensis	812.0	late

formed in SPSS 13.0 for Windows at a significance level of $P<0.05$.

Results

ABA Contents in Leaf Litters The contents of ABA in different plant species' fallen leaves were dramatically different from one another. In Changbai Mountain, the mean content of ABA in the leaf litter of *B. platyphylla* was 1.81 μg/g dw (dry weight), while in the late-successional species (*P. koraiensis*), it was 5.52 μg/g dw, significantly higher than in the pioneer species ($P<0.001$, independent-samples *t*-test). In Tianmu Mountain, the mean content of ABA in the leaf litter of *P. massoniana* was 3.11 μg/g dw compared to 9.66, 4.28, and 16.17 μg/g dw in three mid-successional species (*S. superba, C. gracilis*, and *C. sclerophylla*, respectively), and 13.36 μg/g dw in *C. eyrei*. In Dinghu Mountain, the mean content of ABA in the leaf litter of *P. massoniana* was 3.30 μg/g dw, while the two mid-successional species (*Ca. chinensis* and *C. fissa*) were 11.19 and 4.39 μg/g dw, respectively. The mean content of ABA in two late-successional species (*C. concinna* and *Cr. chinensis*) were measured as 6.06 and 33.67 μg/g dw, respectively (Fig. 1).

In all three forest ecosystems, the early-successional species had the lowest ABA content in their leaf litter. The mid- and late-successional species had significantly more ABA in their leaf litter than did pioneer species, which implies that there is more ABA released into the soil in the middle and late phases of forest succession than in the early phase.

ABA Content in Soils The content of ABA in dry soil increased in all three types of forest as succession progressed. As a micro molecular organic compound, ABA dissolved in soil solution and its concentration also depended on soil moisture. Therefore the concentration of ABA in soil solution also increased with the process of succession, but not universally.

In Changbai Mountain, the mean content of ABA in the soil was 83.69 ng/g dw for the early successional phase, 98.65 ng/g dw for the middle phase, and 121.14 ng/g dw for the late phase (Fig. 2a). The mean concentration of ABA in soil solution was 127.93 ng/ml, 267.48 ng/ml, and 199.11 ng/ml, respectively, for the early, middle, and late successional stages (Fig. 2d). In Tianmu Mountain, the mean content of ABA in the soil increased from 100.1 ng/g dw in the early phase to 210.4 ng/g dw in the early mid-successional phase and to 270.1 ng/g dw in the later mid-successional phase, and to 382.4 ng/g dw in the late-successional phase (Fig. 2b). Accordingly, the mean concentration of ABA in the soil solution increased from 350.38 ng/ml in the early phase to 625.56 ng/ml in the early mid-successional phase and 694.04 ng/ml in the late mid-successional phase, and to 1684.15 ng/ml in the late-successional phase (Fig. 2e). In Dinghu Mountain, the mean ABA content in the soil was 122.0 ng/g dw, 218.5 ng/g dw, 336.6 ng/g dw, and 440.7 ng/g dw in the early, early mid-successional, later mid-successional, and late-successional stages, respectively (Fig. 2c). The mean ABA concentration

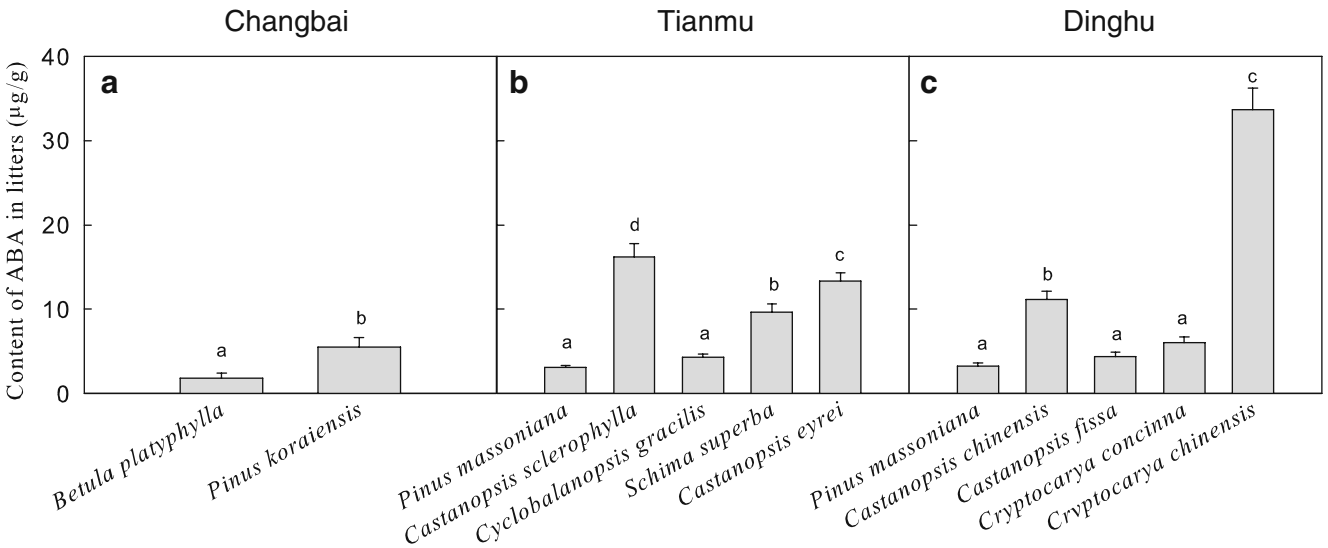


Fig. 1 The content of ABA in the leaf litter of dominant species in the three forest communities. (**a**) Species from Changbai Mountain; (**b**) Species from Tianmu Mountain; (**c**) Species from Dinghu Mountain. Error bars are +/− SE. Means with different letters are significantly different at a significance level of $P<0.05$ (independent-samples *t*-test in Changbai Mountain and one-way ANOVA LSD tests in Tianmu and Dinghu Mountain)

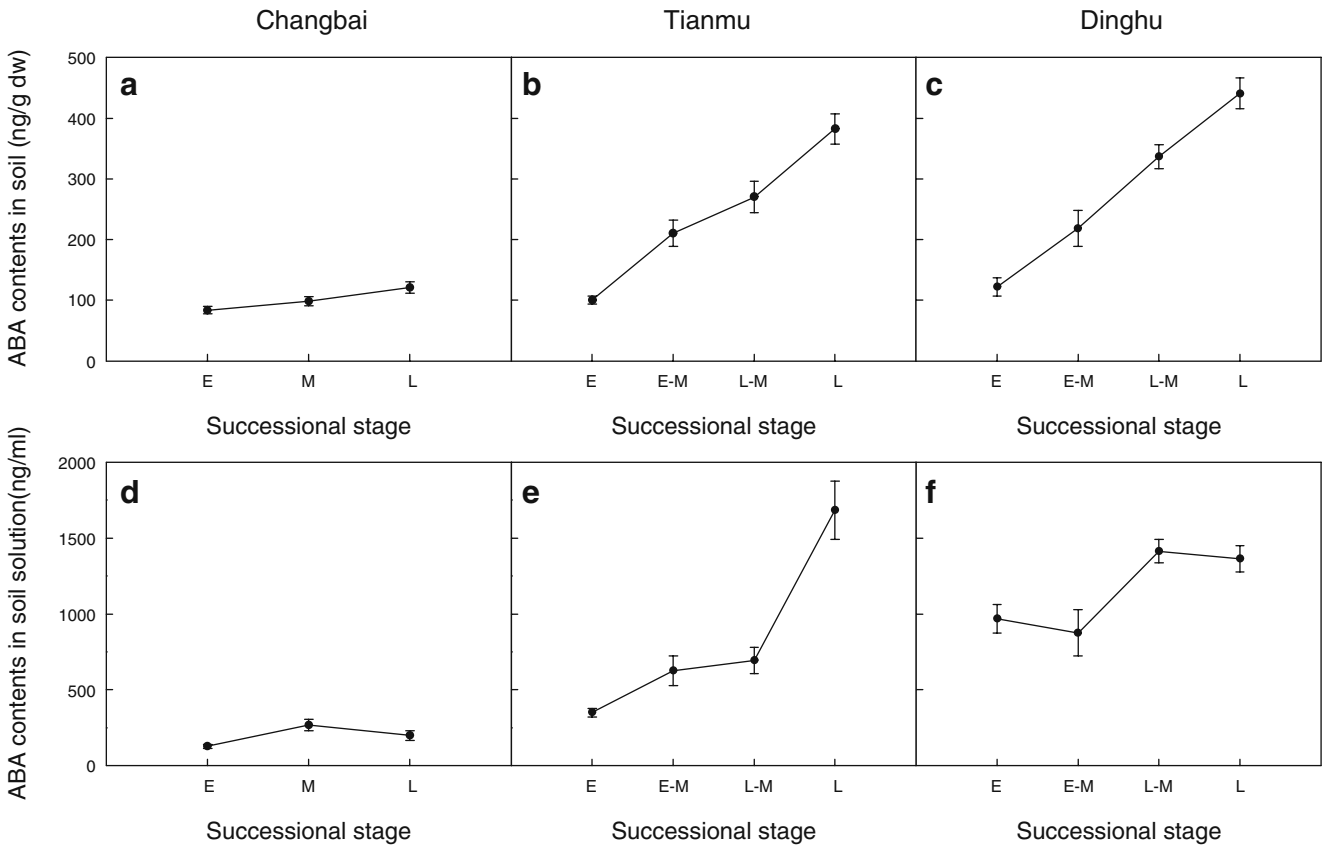


Fig. 2 ABA contents in soil and ABA concentration in soil solution in different successional phases in three forest types. Error bars are +/− SE. The letters below the horizontal axis indicate the successional phases (E, early; E-M, early-middle; M, middle; L-M, late-middle; L, late). (**a**)ABA content in soil in Changbai Mountain; (**b**)ABA content in soil in Tianmu Mountain; (**c**) ABA content in soil in Dinghu Mountain; (**d**) ABA concentration in soil solution in Changbai Mountain; (**e**)ABA concentration in soil solution in Tianmu Mountain; (f)ABA concentration in soil solution in Dinghu Mountain

in the soil solution was 968.74 ng/ml, 875.24 ng/ml, 1413.97 ng/ml, and 1363.80 ng/ml from the early through late successional stages (Fig. 2f).

The northern forest soils had the lowest ABA concentrations of all three forests, in accordance with the observation that species from northern forests have the lowest ABA concentrations in their leaf litter.

It is possible that some of the ABA in the soil may have partially decomposed before being assayed, although we froze the soil samples to minimize this loss. In addition, small amounts of ABA may have been lost in the process of extraction and purification, so the tested concentrations of ABA in the soil are likely even somewhat lower than natural concentrations in field.

Bioassay Seed germination and early seedling growth of pioneer, mid- and late-successional species showed different responses to ABA exposure. ABA had stronger negative effects on pioneer species than on mid- and late-successional species (for detailed dates see Supplementary Table S1 and S2). In the Changbai Mountain forest, seed germination and early seedling growth of *B. platyphylla* was inhibited by ABA at all concentration levels. In contrast, *P. koraiensis* was inhibited only at the highest concentration (8.0 μg/ml) and was influenced only slightly at low concentrations (Fig. 3a and b). In the soil solution, ABA concentrations ranged from 127.93 ng/ml to 267.48 ng/ml. As artificial ABA has less biological activity than natural ABA, this concentration approaches the lowest concentration (0.5 μg/ml) that we tested. At this concentration, ABA had no influence on the late-successional species (*P. koraiensis*) but had an inhibitive effect on pioneer species (*B. platyphylla*). In the Tianmu Mountain forest, the inhibitory effect of ABA on pioneer species was substantially higher than on all other mid- and late-successional species except *S. superba*, which showed higher sensitivity to ABA than pioneer species. The late-successional species *C. eyrei* was affected the least of all five tested species at high concentrations (4.0 and 8.0 μg/ml) (Fig. 3c and d). The mean ABA concentration in soil solution in the early successional stage was 350.38 ng/ml, approaching the lowest ABA concentration cultures. The pioneer species (*P. massoniana*) and one of the mid-

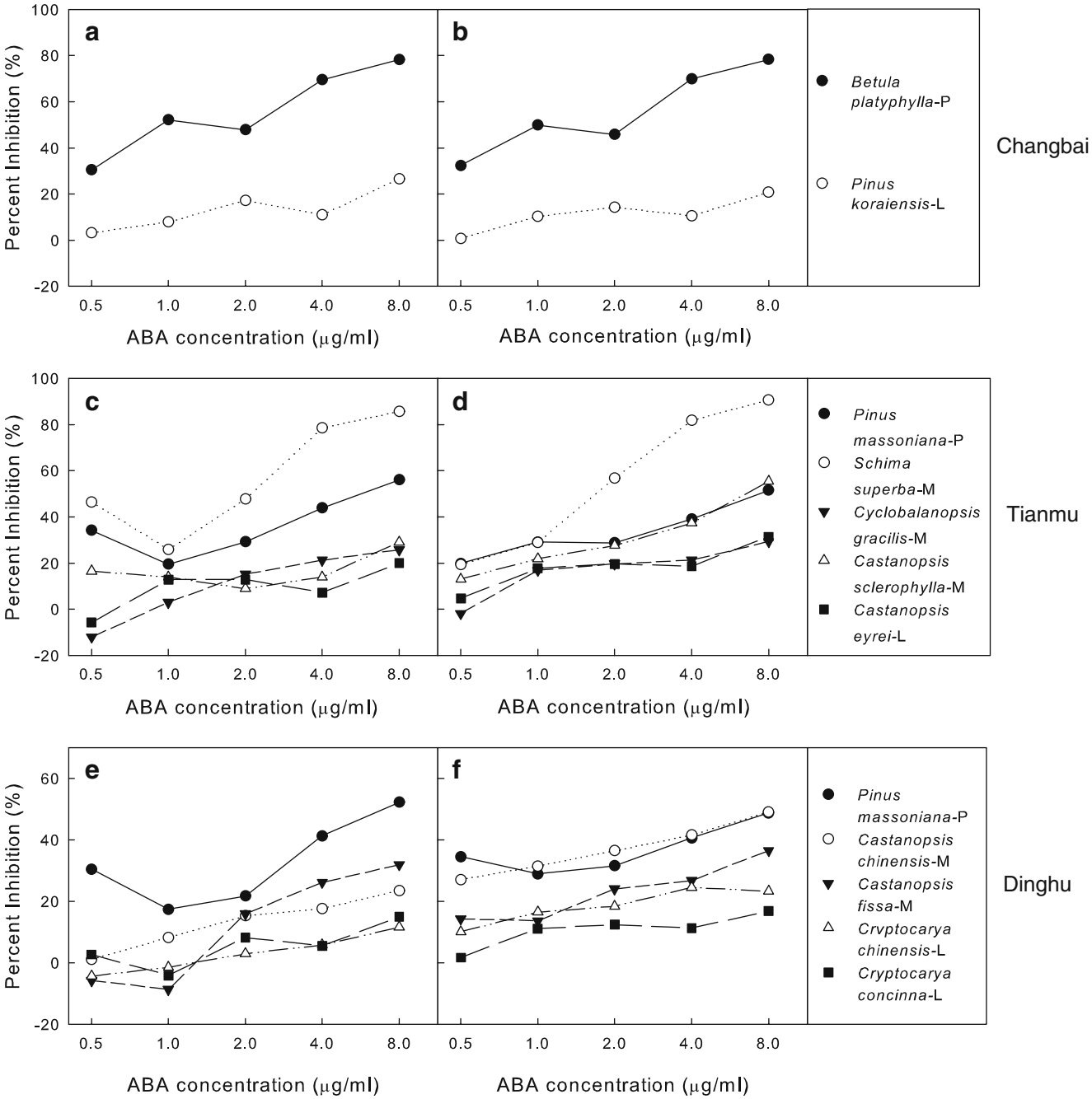


Fig. 3 Inhibitory effects of ABA on the seed germination and early seedling growth of dominant species in three forests. (**a**) Seed germination and (**b**) seedling growth on Changbai Mountain. (**c**) Seed germination and (**d**) seedling growth on Tianmu Mountain. (**e**) Seed germination and (**f**) seedling growth on Dinghu Mountain. P = pioneer species; M = midsuccessional species; L = late-succesional species

successional species (*S. superba*) were inhibited at this concentration, but mid- and late-successional species were influenced only slightly. In mid- and late-successional stages, ABA concentrations in the soil solution increased, and pioneer and mid-successional species were more inhibited; the late-successional species also was inhibited but to a lesser degree than the other species.

In the Dinghu Mountain forest, seed germination of *P. massoniana*, an early-successional species, showed the strongest suppression by ABA at all concentrations. Mid-successional species had the same response to ABA at low concentrations (0.5 and 1.0 μg/ml) as late-successional species, but had a more negative response at high concentrations (>2.0 μg/ml). The seed germination and

early seedling growth of late-successional species at Dinghu Mountain were least inhibited of the three forests, and had no significant response to ABA at any concentration level (Fig. 3e and f). In the soil solutions, ABA concentrations approached 1.0 μg/ml in early-successional stage forests and 2.0 μg/ml in late-successional stage forests. Pioneer and some mid-successional species were inhibited at all successional stages, but the other mid- and all late-successional species were not influenced in the early stage and were only slightly inhibited in later stages.

There was a significant negative correlation between seed mass and inhibition of ABA on seed germination ($P<0.01$ at all concentrations) and early seedling growth ($P<0.05$ at all concentrations) in all 12 species tested, indicating that small seeds are more sensitive to the inhibitory effects of ABA than large seeds. Additionally, inhibition of seed germination by ABA was more closely related to seed mass than early seedling growth, which indicates that the process of seed germination under ABA suppression is reflected by seed mass.

Discussion

Plant litter can alter the physical and chemical environment of a community and affect the probability of germination and establishment of plants (Facelli and Pickett 1991a; Bartha, 2001). The negative effects of litter on seedling recruitment have been recorded in many species (Goldberg and Werner, 1983; Facelli and Kerrigan, 1996; Foster, 1999; Vellend et al., 2000). During the process of succession, litter fall increases with increasing age of vegetation (Jensen and Gutekunst, 2003; Ostertag et al., 2008). Species from different successional stages may respond differently to litter cover. Monk and Gabrielson (1985) found that pioneer species were more affected by litter than late successional species. Multiple mechanisms, including light interception (Facelli and Pickett, 1991a, b; Jensen and Gutekunst, 2003), reduction of the amplitude of temperature (Grime et al., 1981), mechanical effects (Bosy and Reader, 1995), and facilitation of seed predators (Facelli, 1994) may be involved in the effects of litter on seed germination and seedling establishment. Allelochemicals leached from litter also have been considered as one of the mechanisms of succession (Facelli and Pickett, 1991a, b; Ganade and Brown, 2002). In this study, we found a very high concentration of ABA in forest leaf litter, which may be one of the reasons that litter has an important suppression effect on plant establishment. The concentration of ABA in grass leachates of tall fescue and straw had previously been measured at 390 ng/g fw (Buta and Spaulding, 1989). ABA concentrations in the leaf litter of the 12 species tested here were much higher than those previously recorded. ABA content also had been reported to vary greatly between fresh and dry leaves. A 10-fold increase in ABA levels had been observed in grass after one day of desiccation (Buta and Spaulding, 1989). Therefore, it is not unusual that the fallen leaves measured here had such high ABA concentrations.

During the course of succession, leaf traits such as leaf area, matter content, and chemical composition change as forest plant community composition and plant life form dominance change (Cortez et al., 2007). Most theories of plant herbivory predict that late-successional species produce greater amounts of chemical defense compounds for protection from desiccation, herbivory, and parasitism (e.g., Coley et al., 1985). Our results show that late-successional species produce more ABA in their fallen leaves than early-successional species. This could alter the chemical environment of the community and help later-successional species gain a competitive advantage over early-successional species by inhibiting the germination and growth of early-succesional species. In this way, ABA may serve as a chemical weapon of interspecific competition.

Leaf litter is the main source of ABA in the soil. As community succession progresses, both litter production and the amount of ABA in the litter increase. However, decomposition by soil microorganisms also increases with succession. Therefore, the true ABA concentration is a balance between ABA loss and retention in the soil; overall, the concentration increases as succession progresses. In maize, 10 nM (2.64 ng/ml) of ABA has been found to have an inhibitory effect on root elongation (Pilet and Saucy, 1987). In our study, the concentrations of ABA in the soil were much higher than levels shown previously to have inhibitory effects on plant species.

Soil is an important factor that influences plant community composition, and soil abiotic and biotic factors play key roles in plant community dynamics. Kardol et al. (2006) observed negative plant–soil feedback for early-successional plant species, neutral feedback for mid-successional species, and positive feedback for late-successional species. Many secondary compounds accumulate in soil organic matter (e.g., Koegel-Knabner et al., 1991; Northup et al., 1998; Hattenschwiler and Vitousek, 2000) and alter the chemical environment of the community. The accumulation of ABA in soil represents one possible chemical factor associated with community succession. The changed soil environment likely will directly inhibit some plant species while facilitating others, and ultimately influence community composition and vegetation succession.

Seed germination and early seedling growth are perhaps the most vulnerable stages in the life cycle of many plant species (Hester et al., 1991), and the suppression of

seedling recruitment can have serious consequences for population viability and species diversity (Tilman, 1993). ABA has been widely documented as an inhibitor of seed germination and early seedling growth in many species (Amaral et al., 2008), although its inhibitory effect varies among species (Buta and Spaulding, 1989; Tseng et al., 2003). In this study, the inhibitory strength of ABA depended on the successional stage at which the species occurred. ABA strongly inhibits both seed germination and the early growth of seedlings of pioneer species, but has little impact on mid- and late-successional species. This difference among species can change the competitive relationship between early- and late-successional species.

The mature stages of the life cycles of pioneer and late-successional species also may have different responses to the inhibitory effects of ABA, but this is not addressed in this paper. Tolerance of higher ABA concentrations in the initial stages of the life cycle would give late-successional species an additional advantage compared to pioneers. In the process of succession, increasing ABA concentrations in the soil as well as simultaneous changes among other physical and biological factors could cause pioneer species to lose their competitive advantage and be replaced by later-successional species.

ABA also can have a promotive effect on seed germination and plant growth when applied at low concentrations, a phenomenon known as hormesis (Mowha and Jackson, 1976, Chobot and Hadacek, 2009). In this study, seed germination of *C. gracilis, C. eyrei, C. fissa*, and *Cr. chinensis* were slightly stimulated by low concentrations of ABA. This implies that toxic levels of ABA for pioneer species may actually be favorable for late-successional species.

Early successional species often have a lower seed mass than late-successional plants (Jensen and Schrautzer, 1999; Jensen and Gutekunst, 2003), and this also was observed in this study. Seed size is a key feature for the regeneration of plants and affects both competitive and colonizing abilities of the species (Turnball et al., 1999; Jensen and Gutekunst, 2003). Large seed size correlates with higher amounts of nutrient storage, and large initial size has been shown to be an advantage for germination and establishment under shaded conditions and beneath litter layers (Venable and Brown, 1988; Leishman and Westoby, 1994; Jensen and Gutekunst, 2003). In our study, large seeds tolerated the inhibitory effect of ABA better than small seeds. Small seeds have little nutrient storage and must synthesize them through photosynthesis to provide for early growth. We hypothesize that ABA can inhibit the synthetic system to regulate seed germination and early seedling growth. Small seeds cannot gain nutrients in the presence of ABA and are thus inhibited. Conversely, nutrient storage in large seeds may help them pass this ABA-sensitive stage of life cycle. This theory explains why large seeds can germinate and grow well under thick litter and in soils with high ABA concentrations. Of course, the mechanism of large seeds' ability to tolerate high ABA levels needs investigation.

Our findings indicate that ABA accumulates in the soil during plant community succession and changes the chemical environment of the community. Increasing ABA concentrations in soil, associated with other community and species features, likely suppress the establishment of early-successional species and indirectly facilitate subsequent species, which, over time, leads to plant community succession. This finding has probable significance for forest management and ecological restoration.

Multiple deterministic and stochastic processes determine plant succession, and the detailed prediction of successional trajectories remains elusive. For example, although our results can help to explain why pioneer species are replaced by late-successional species, they do not explain why late-successional species can invade an established pioneer community. Our work, however, provides important initial research on the importance of ABA in community succession, and highlights the need for more research to be done in this area. We still lack an understanding of the effects of ABA in soil solutions over a longer time period, and additional sources of ABA, including plant root and stem radial flow, need to be detailed. ABA may influence not only seed germination and seedling growth, but also affect plant growth and interspecies relationships beyond the seed stage. Thus, the role of ABA accumulation in plant succession merits further investigation.

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Smelling the Wood from the Trees: Non-Linear Parasitoid Responses to Volatile Attractants Produced by Wild and Cultivated Cabbage

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Abstract Despite a large number of studies on herbivore-induced plant volatiles (HIPVs), little is known about which specific compounds are used by natural enemies to locate prey- or host- infested plants. In addition, the role of HIPVs in attracting natural enemies has been restricted largely to agricultural systems. Differences in volatile blends emitted by cultivars and plants that originate from wild populations may be attributed to potentially contrasting selection regimes: natural selection among the wild types and artificial selection among cultivars. A more realistic understanding of these interactions in a broader ecological and evolutionary framework should include studies that involve insect herbivores, parasitoids, and wild plants on which they naturally interact in the field. We compared the attractiveness of HIPVs emitted by wild and cultivated cabbage to the parasitoid *Cotesia rubecula,* and determined the chemical composition of the HIPV blends to elucidate which compounds are involved in parasitoid attraction. Wild and cultivated cabbage differed significantly in their volatile emissions. *Cotesia rubecula* was differentially attracted to the wild cabbage populations and preferred wild over cultivated cabbage. Isothiocyanates, which were only emitted by the wild cabbages, may be the key components that explain the preference for wild over cultivated cabbage, whereas terpenes may be important for the differential attraction among the wild populations. Volatile analysis revealed that parasitoid attraction cannot be explained by simple linear relationships. Our results suggest that unraveling which compound(s) are innately attractive to parasitoids of cabbage pests should include wild Brassicaceae.

Key Words *Brassica* · *Cotesia rubecula* · HIPV· Induction · Jasmonic acid · Terpenes · Terpenoids · Tritrophic interaction · VOC

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Introduction

The study of the factors that underpin rules governing the assembly, structure, and function of ecological communities is complicated by the fact that natural systems generally are characterized by a complex array of structural and chemical heterogeneity. This heterogeneity poses a profound challenge in the exploitation of resources by consumers (herbivores and their natural enemies). Individuals that use information enabling them to exploit resources will enjoy fitness benefits (e.g., Vet and Dicke 1992). Plants are known to emit volatile blends that provide information about the plant's identity, and can provide consumers with reliable information about the presence of a plant that might be a source of food (for herbivores), hosts (for parasitoids), or prey (for predators). However, as volatile signals do not always reveal which prey or host is feeding on the emitting plant and are released in a heterogeneous background, there will be variation in how this information is utilized by organisms throughout the food chain.

The functional and ecological importance of herbivore-induced plant volatiles (HIPVs) that are utilized by predators and parasitoids of herbivorous arthropods has received considerable attention over the past 25 years (e.g., Sabelis and Van de Baan 1983; Dicke et al. 1990; Turlings et al. 1995; De Moraes et al. 1998; Dicke and Baldwin 2010). However, despite the large number of papers on this topic, little is known about which specific plant compounds or mixtures of compounds are used by natural enemies to locate prey- or host- infested plants. Many studies with parasitoids of herbivorous arthropods have shown that experienced female wasps, i.e., wasps that have been in contact with hosts or the food plant of the host, are able to discriminate between volatile blends emitted by undamaged and herbivore-damaged plants, and that they prefer the latter (reviewed by Steidle and van Loon 2003; Turlings and Wäckers 2004; Allison and Hare 2009).

Study of the importance of specific HIPVs in natural enemy attraction traditionally has focused on terpenes, fatty acid derivatives (especially those produced by the lipoxygenase pathway), and products from the phenylalanine lyase (PAL) pathway (Ozawa et al. 2000; Schnee et al. 2006; Shiojiri et al. 2006a; Halitschke et al. 2008; Zhang et al. 2009). For example, specific terpenes attract natural enemies of arthropod herbivores when offered individually (Dicke et al. 1990; Rasmann et al. 2005) and are perceived by receptors in the antennae (Smid et al. 2002; Gouinguene et al. 2005). However, Mumm et al. (2008) showed that treatment of cabbage plants with fosmidomycin, an inhibitor of the biosynthesis of certain monoterpenes, did not affect the behavior of *Cotesia glomerata*. This throws doubt on the role of these monoterpenes as parasitoid attractants in this cabbage-parasitoid interaction.

Research on HIPVs and their role in host searching behavior of parasitoids has been driven largely by the potential for insect parasitoids as biological control agents of insect pests. Consequently, these studies often are restricted to cultivated plant species (Berenbaum and Zangerl 2008). However, artificial selection for specific plant traits has often not only changed the appearance of cultivated plants compared to their wild conspecifics, but also their chemistry. For example, wild cabbage *Brassica oleracea* populations, which grow naturally along the Atlantic coastline of western Europe, contain significantly higher levels of glucosinolates than cultivated cabbage (Harvey et al. 2007; Gols et al. 2008a). Glucosinolates (hereafter GS) are secondary plant compounds that play an important role in defense against insect herbivores and pathogens (Rask et al. 2000). However, the effect of domestication on the volatile profiles of plants has been little studied. For example, it is not known if HIPVs emitted by cultivated plants are similar to those emitted by wild conspecifics or whether natural enemies are equally attracted to HIPVs produced by cultivated and wild conspecifics. Given that insect predators and parasitoids have evolved in natural communities, this is a major gap in our knowledge. Köllner et al. (2008) have shown that a terpene synthase (TPS23) is involved in the production of (*E*)-β-caryophyllene, an HIPV that attracts natural enemies of a maize herbivore (Rasmann et al. 2005; Köllner et al. 2008). This gene is expressed in European maize lines as well as in its closest wild relative teosinte, but not in most American cultivated maize varieties (Köllner et al. 2008). This finding suggests that, in addition to plant traits that affect herbivory directly, volatile emissions that can be used by an herbivore's natural enemies also may differ between cultivated and natural conspecific plants.

Here, we compared the chemistry and attractiveness of HIPV blends emitted by plants originating from three wild cabbage (*Brassica oleracea* L, Brassicaceae, Fig. 1) populations, which differ dramatically in their levels of secondary plant metabolites (Gols et al. 2008b). Consequently, we expected variation in volatile secondary metabolites in response to herbivory, which in turn would differentially attract a parasitoid specialized on hosts feeding on brassicaceous plants. In addition, we compared the attractiveness of HIPV blends emitted by wild and cultivated cabbage to investigate the effect of domestication on parasitoid attraction. Our results are discussed in the context of the phenology of multitrophic interactions in natural communities, an aspect that is often ignored in studies of plant-insect interactions. We argue that studies examining the role of HIPVs on host searching behavior in parasitoids should explore more biologically and ecologically realistic interactions that involve herbivores and their parasitoids on wild food plants, on which these multitrophic interactions initially evolved.



Fig. 1 Wild *Brassica oleracea* growing naturally along the south coast of Dorset, England

Methods and Materials

Plants Seeds of *Brassica oleracea* were collected from several plants (>15) in three wild cabbage populations growing within 20 km of each other on chalk cliffs along the south coast of England, near Swanage in Dorset. These wild cabbage populations generally are regarded as the native wild form of cultivated cabbage (Wichmann et al. 2008). The three wild populations were located at sites known as 'Old Harry' ('OH', 50°38′N, 1°55′E), 'Kimmeridge' ('KIM', 50° 35′N, 2°03′E), and 'Winspit' ('WIN', 50°36′N, 2°07′E). The cultivar used in the experiment was a Brussels sprout variety (*B. oleracea* gemmifera) cv. Cyrus (CYR hereafter) and differs quantitatively and qualitatively in GS chemistry compared to the three wild cabbage populations (Gols et al. 2008b). Seeds were germinated and seedlings transferred to 1.5-l pots filled with potting soil ('Lentse potgrond #4'). Plants were grown in a greenhouse at 20–30°C, 40–80% r.h., and a photoperiod of at least 16 hr. Plants were watered daily. When plants were 4-wk-old, they were fertilized once a week with Kristallon blauw (N: P: K: micro nutrients as 19: 6: 20: 4) at 2.5 mg ml^{-1}, which was applied to the soil. Plants were 6-wk-old when they were used in experiments.

Insects The herbivore *Pieris rapae* L (Lepidoptera: Pieridae) and its specialist parasitoid *Cotesia rubecula* Marshall (Hymenoptera: Braconidae) are native to the Palearctic realm. In the Netherlands, *P. rapae* has three generations per year. In spring and early summer, the larvae feed mainly on GS-containing wild brassicaceous plant species (Fig. 2), whereas in late summer and autumn, cultivated species also may become important food plants. In this study, the herbivore and the parasitoid were collected originally from a Brussels sprouts field in the vicinity of Wageningen, The Netherlands. Insects were maintained in climate-controlled rooms at 20–24°C, 60% r.h., and a photoperiod of 16:8 h L:D. They were reared on cultivated CYR plants for at least 5 generations. Parasitized hosts were reared on CYR plants until the parasitoids had completed their immature development and egressed from the host. At this point, parasitoid cocoons were collected and transferred to a clean insect cage until adult emergence. Adult wasps were provided with honey as an energy source. In the flight bioassay, we used mated females that were 4–5-d-old and that had had no previous experience with hosts and are referred to as being 'naïve'. Wasps were used only once in the experiments.

To induce the plants, we used first instar (L1) *P. rapae* larvae, which were obtained from the insect culture.

Attraction of C. Rubecula to Volatiles Emitted by Wild and Cultivated Cabbage Experiment 1 – Herbivore-induced wild cabbage plants. The flight response of female *C. rubecula* to plants infested with *P. rapae* was tested in a tent-like structure (330×175×280 cm) made out of white nylon sheets, which was placed in a greenhouse (22–25°C, 50–70% r.h.). The greenhouse was continuously ventilated, which created a turbulent air current inside the tent. Previous studies have reported that 24 hr of caterpillar feeding results in the production of HIPV (Geervliet et al. 1997; Bruinsma et al. 2009). Each plant was infested with

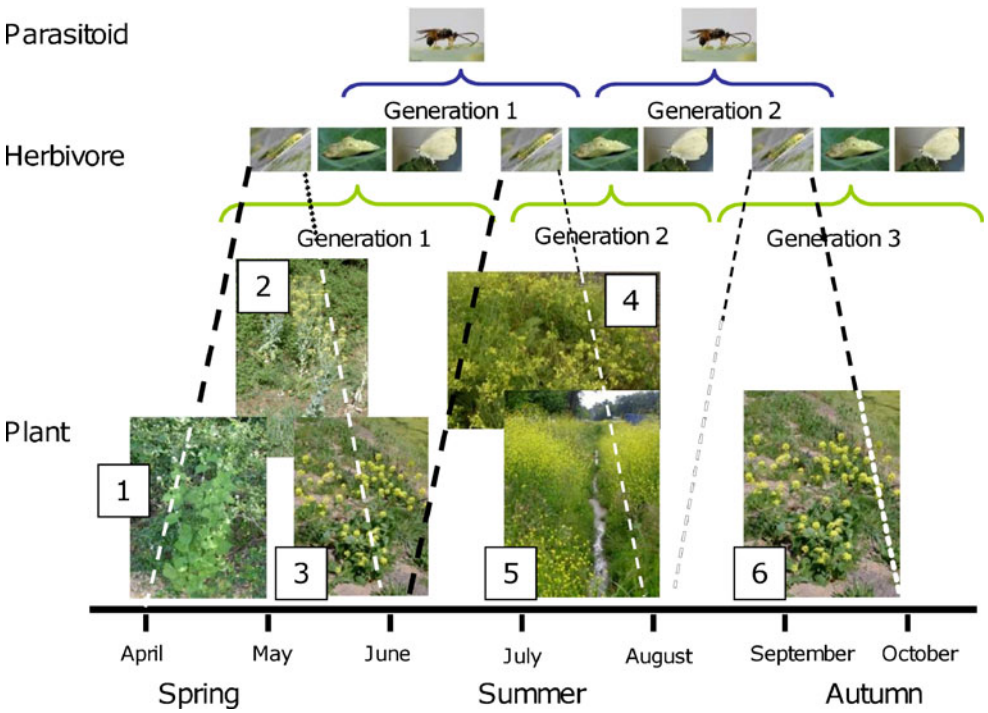


Fig. 2 Phenology of various wild food plants of *Pieris rapae* and occurrence of different generations of this herbivore and its specialist parasitoid *Cotesia rubecula* in the Netherlands. (1) *Alliaria petiolata*, (2) *Brassica napus*, (3) *Sinapis arvensis* (1st generation), (4) *Raphanus raphanistrum*, (5) *Brassica nigra*, (6) *S. arvensis* (2nd generation)

15 L1 *P. rapae* larvae divided over 3 leaves 20–24 hr prior to the bioassay.

We tested whether female wasps were equally attracted to plants of the 3 wild *B. oleracea* populations. Three host-infested plants, one from each cabbage population, were placed in a triangle 1 m apart. *Cotesia rubecula* females were collected from the insect cage in glass vials that were closed with cotton wool. These vials with single wasps then were placed in the center of the set-up, and the cotton wool was removed. Since the wasps did not leave the vial immediately, up to 5 vials were placed on the table simultaneously. Wasps were allowed to fly freely within the tent. As soon as a wasp landed on one of the three plants, it was collected, and the plant's identity was recorded. If some of the wasps that were initially released had not alighted on one of the plants within 30 min, they were excluded from the analysis. The response level, i.e., the proportion of wasps that alighted on a plant out of the total of wasps released, varied between 70 and 95%. A total of 30 responsive wasps were recorded for each combination of 3 plants. Based on visual inspection, each plant in a tested combination of plants had received similar amounts of herbivore damage. To control for any bias caused by the position of the plants, individual plants were rotated one position after 10 wasps had landed. The bioassay was repeated 14 times with newly prepared plants over 5 weeks.

Experiment 2 – Herbivore-induced plants: cultivated vs. wild cabbage. Here, we tested whether female *C. rubecula* were equally attracted to the Brussels sprout cultivar (CYR) compared to one of the wild cabbage populations (OH). Plant individuals were infested with 15 L1 *P. rapae* larvae divided over 3 leaves 20–24 hr prior to the bioassay. Two host-infested plants, one CYR and one OH plant were placed at opposite ends of the table (1 m apart) in the tent. Single wasps were released in vials half-way between the two plants. Observations were similar to the previous experiment. A total of 12 responsive wasps were recorded for each combination of two plants. To control for any positional bias, the positions of the plants were switched after 6 wasps had landed on the plants. The bioassay was repeated 15 times with newly prepared plants over 5 weeks.

Experiment 3 – Jasmonic acid (JA)-induced wild cabbage plants. The production of HIPVs is regulated by at least three signal-transduction pathways, the jasmonic acid (JA), the salicylic acid (SA), and the ethylene pathway (reviewed by Arimura et al. 2009). Biosynthesis of volatiles induced by biting-chewing insect herbivores depends mainly on the JA pathway (Reymond et al. 2004; De Vos et al. 2005). Exogenous JA-treatment often is used to mimic the effects of feeding by biting-chewing insects (Dicke et al. 1999; Thaler 1999; Bruinsma et al. 2009; Zhang et al. 2009). Here, we compared JA-treated plants from the 3 wild populations to investigate whether differential attraction to HIPV as observed in experiment 1 can be explained by differences in volatile emissions, whose biosynthesis is dependent on JA signalling.

Plants were sprayed with 15 ml of 1 mM JA (± jasmonic acid Sigma-Aldrich >97% purity) in a 0.2% Tween 80 (Merck) aqueous solution 20–24 hr prior to experiments. Control plants were sprayed with 15 ml of 0.2% Tween 80 in water. When plants were treated with JA, few wasps (<20%) initiated flying in the set-up that was used in the previous two experiments. Therefore, the flight response of *C. rubecula* was recorded in a wind tunnel (details in Geervliet et al. 1994), because a previous study showed that the wasps do respond to JA-treated cabbage plants in this device (Bruinsma et al. 2009). The wind tunnel conditions were set at 24±2°C, 60–70% r.h., and a wind speed of 20 cm s^{-1}. First, we investigated whether JA-treated OH plants attract more wasps than control OH plants. To investigate whether plants from the three wild populations are equally attractive to *C. rubecula* when treated with JA, three pair-wise comparisons were made, OH-WIN, OH-KIM, and WIN-KIM, as the wind tunnel only allows for dual-choice comparisons. Up to 5 vials with single wasps were placed in a horizontal glass cylinder on a 10-cm high socket positioned 60 cm downwind from the two plants. Recordings were similar to the previous experiments. Wasps that had not alighted on one of the plants within 15 min were removed and excluded from the analysis. Additional wasps were released until a total of 12 wasps had made a choice. The response level of the females in the wind tunnel varied between 60 and 90%. The position of the plants was switched after 6 wasps had made a choice. Each plant combination was tested eight times with newly prepared plants over 6 weeks.

Headspace Analysis of Herbivore Infested Plants: Collection of Volatiles Volatiles were collected from individual host-infested plants (N=5 or 6) from each of the four *B. oleracea* lines used in the bioassays. Plants were induced similarly as in the flight bioassays, i.e., plant individuals were infested with 15 L1 *P. rapae* larvae divided over 3 leaves 20–24 hr prior to volatile collection An entire host-infested plant, including soil, which was contained in aluminum foil, was transferred to a clean 35-l glass collection container. Pressurized air was filtered over silica gel, molecular sieves (4 Å, Sigma, St Louis MO, USA) and activated charcoal before entering the sampling container. Prior to sampling, the container was purged for 1 hr at 10 l/h. Subsequently, headspace volatiles were trapped on 90 mg Tenax TA for 4 hr at a flow of approximately 40 ml/min. Volatiles were also collected (N=2) from a collection container containing a piece of aluminum foil with water. Volatile compounds detected in these control samples were excluded from the data obtained for the plant samples.

Collected volatiles were released from the Tenax traps by heating in a thermodesorption cold trap unit (Markes, UK) at 200°C for 10 min and flushing with a He flow of 30 ml/min. Released compounds were cryofocused in a coldtrap at a temperature of −10°C. Volatiles were injected in splitless mode into the RTX-5 Silms GC column (Restec, 30 m×0.32 mm ID, 0.33 μm film thickness) by heating of the cold trap to 270°C. After an initial column temperature of 40°C for 2 min, the temperature was raised to 90°C at 3°C/min, then to 165°C at 2°C/min, and subsequently to 250°C at 15°C/min. The column was directly coupled to the ion source of a Finnigan quadrupole mass spectrometer, which was operating in the 70 eV EI ionization mode and scanning from mass 33 to 300 at 3 scans/sec. Collected volatiles were separated and identified using GS/MS (gas chromatography combined with mass spectrometry). Compounds were tentatively identified by comparison of the mass spectra with those in the NIST 2002 (version 2a) and Wiley 7 edition spectral libraries and by checking the retention indices. For identification and quantification, we used AMDIS version 2.1 (Automated Mass spectral Deconvolution & Identification Software). Compounds were quantified by measuring peak areas of the total ion fractions of the integrated signal. Peak areas were divided by above-ground fresh weight of plant tissue, which was determined immediately after trapping.

Statistical Analysis To determine whether *C. rubecula* females were differentially attracted to the three wild cabbage populations, we conducted a *G*-test for independence on wasp counts in the replicated three-way choice bioassays. The results for the replicate tests were homogeneous, so a *G-test* (goodness of fit) was conducted on the pooled data (H_0 = the three populations are equally attractive). A similar approach was used for the dual choice bioassays.

First, we determined whether population preferences could be explained by abundance patterns of individual volatile compounds. Each of the volatiles produced by the three wild populations was subjected to Kruskal-Wallis non-parametric one-way ANOVA. For the CYR-OH comparison, *Mann–Whitney U* tests were used. For each of the compounds for which the population effect was significant, we determined whether the emissions quantities ranked according to the landing preference. In a second approach, we used multivariate statistics (Principle Components Analysis = PCA) to reveal: (1) which volatile compounds are important for the separation of the three wild cabbage populations; (2) which are important for the separation of the OH and WIN plants; and (3) which are important for the separation of the wild OH and cultivated CYR plants. PLS-DA (Projection to Latent Structures Discriminant Analysis) is a projection method that separates groups of observations by rotating the PCA's such that a maximum separation among classes, here *B. oleracea* populations, is obtained (Eriksson et al. 2006). To achieve this, a Y-data matrix of dummy variables is included, which assigns a sample to its respective class, here population. The PLS-DA extension of the SIMCA P+ 12.0 software program (Umetrics AB, Umeå, Sweden) used for this analysis approximates the point 'swarm' in X (matrix with volatile compounds) and Y in PLS components in such a way that maximum covariation between the components in X and Y is achieved. The results of the analysis are visualized in score plots, which reveal the sample structure according to the model components, and loading plots, which display the contribution of the variables to these components, as well as the relationships among the variables. The program's cross validation procedure evaluates the significance of each additional component (starting with none) by comparing the goodness of fit (R^2) and the predictive value (Q^2) of the extended model with that of the reduced model. In a second PLS-DA analysis, those compounds were eliminated that contributed little to the separation of the samples belonging to different populations based on the variable importance on projection values (VIP values). Variables with VIP values greater than 1 are most influential to the model (Eriksson et al. 2006). Per sample, amounts of the individual volatile compounds were measured as peak areas divided by fresh mass (g) of aboveground plant tissues. In the analysis, those volatile compounds were included that were present in at least three of the samples in one of the populations. Based on their biosynthetic pathways, the substances found in volatile plant emissions in response to herbivory can be classified into terpenes, fatty acid derivatives, phenylpropanoids, benzenoids, and various nitrogen and sulfur containing products (Dudareva et al. 2004). Here, we also included the total amounts of alcohols, esters, aldehydes, ketones, sulfides, terpenes, and nitrogen-containing volatiles, as variables in the analyses. Data were log-transformed, mean-centered, and scaled to unit variance before they were subjected to the analysis.

Results

Attraction of C. rubecula to Caterpillar-Induced Volatiles Emitted by Wild and Cultivated Cabbage Plants (Experiments 1 and 2) The results for the flight bioassays in experiment 1 and 2 were homogenous (wild cabbage populations: $\chi 2$=25.2, *df*=26, *P*=0.51; OH vs. CYR plants: $\chi 2$=0.94, *df*=14, *P*=0.95). *Cotesia rubecula* females were differentially attracted to the plants of the three wild populations ($\chi 2$=7.04, *df*=2, *P*=0.03, Fig. 3), and OH plants were significantly more attractive to the parasitoids than

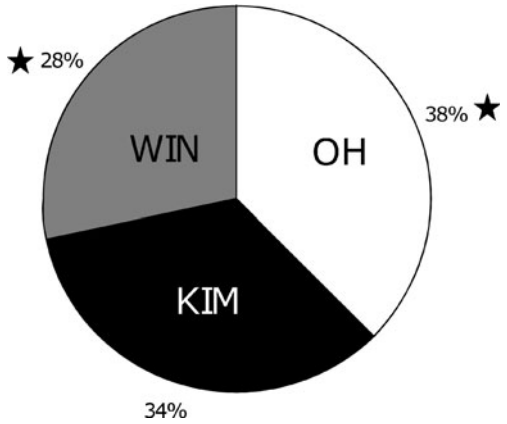


Fig. 3 Landing preference of *Cotesia rubecula* in a three-way comparison of wild host-infested *Brassica oleracea* plants originating from three wild populations (OH, KIM, and WIN) in Dorset, UK. Pie parts represent the average percentages of 14 bioassays performed in a tent set-up. In each bioassay 30 landing preferences were recorded (in total 420 wasps were released). The asterisks indicate populations for which the wasp preference was higher or lower than the expected preference of 1/3 (*G-test* for goodness of fit)

WIN plants (Fig. 3). Despite their rearing history on CYR plants, *C. rubecula* females preferred to alight on OH over CYR plants ($\chi 2=9.67$, $df=1$, $P=0.002$, Fig. 4a)

Attraction of C. rubecula to Volatiles Induced by JA Treatment (Experiment 3) To investigate whether volatiles whose biosynthesis is dependent on the JA signaling pathway are involved in the differential attraction of *C. rubecula*, plants from the three wild populations were treated with JA. In the first set of experiments, OH plants treated with JA were more attractive than OH plants treated with control solution ($\chi 2=6.06$, $df=1$, $P=0.02$, Fig. 4b). However, parasitoids did not discriminate between JA-treated plants of the three populations when offered pair-wise combinations (Fig. 4b).

Headspace Analysis of Herbivore Infested Plants Forty-nine different compounds were detected in at least three samples of one plant population (Table 1). Interestingly, volatile breakdown products of GS (but-3-enyl isothiocyanate and prop-2-enyl isothiocyanate) were produced only by wild cabbage plants. Volatile quantities of 13 compounds, i.e., but-3-enyl isothiocyanate and 12 terpenes, were significantly different when the three wild populations were compared using the *Kruskal Wallis test*. However, none of their emission quantities ranked OH>KIM>WIN, i.e., the preference ranking of *C. rubecula*. Emissions of δ-3-carene ranked WIN>KIM>OH, suggesting that this compound might repel *C. rubecula*. A PLS-DA including volatiles emitted by the three wild cabbages that significantly explained some of the variance, i.e., those compounds that had VIP values >1 (23 compounds), resulted in a model with three significant principal components (PC). The first PC separated KIM samples from the OH and WIN samples, whereas the second PC further separated the OH and the WIN samples (Fig. 5). This means that the volatile blends emitted by OH and WIN plants were more similar to each other than to the blends emitted by KIM plants. The KIM plants were characterized by relatively high emissions of the alcohols 3-pentanol and (*Z*)-3-hexen-1-ol and low amounts of the volatiles located at the right hand side in

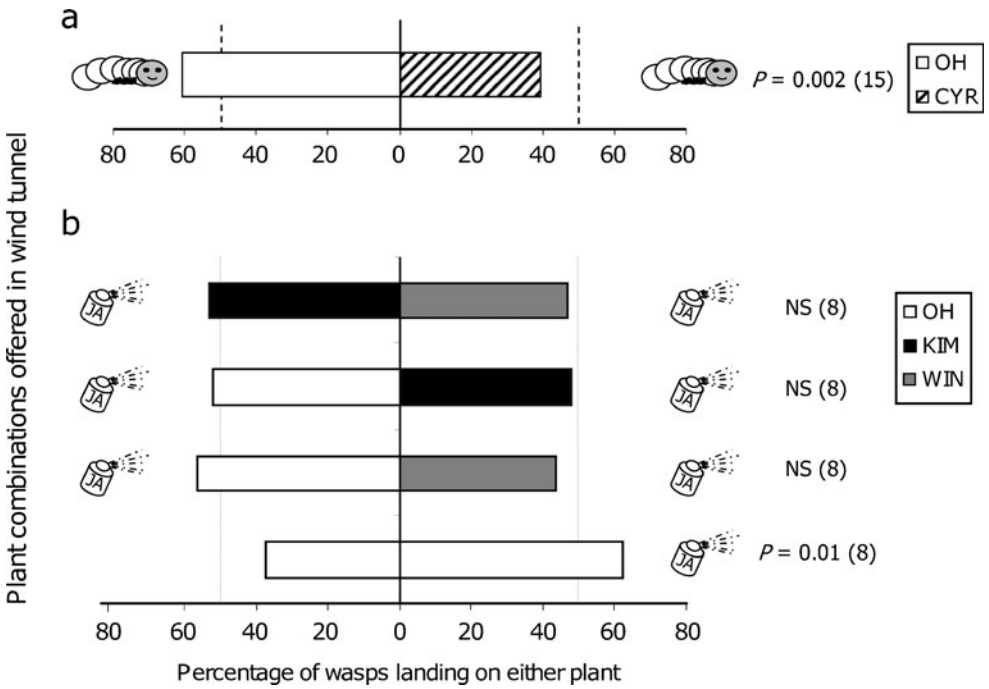


Fig. 4 Landing preference of *Cotesia rubecula* in a two-way comparison with cabbage plants. **a** Comparison of a host-infested cultivated (CYR) and wild (OH) plant and **b** pair-wise comparisons of JA-treated (1 mM) plants of the three wild *Brassica oleracea* populations, OH, KIM, and WIN (top three bars) and OH plants treated with JA or control solution (bottom bar). Experiments were performed in a wind tunnel. Significance levels indicate whether the preference for either plant offered in the bioassay is significantly different from a 50–50 distribution (*G-tests* for goodness of fit, NS = not significant). Numbers in brackets indicate the number of replicates. In each replicate 12 responding wasps were recorded

Table 1 Volatile emissions[a] by plants originating from three wild cabbage (*Brassica oleracea*) populations (OH, KIM and WIN) and a brussels sprout cultivar (CYR) in response to *Pieris rapae* caterpillar feeding

ID[b]	Population → Compound names↓	OH (*N*=6)	KIM (*N*=6)	WIN (*N*=5)	CYR (*N*=5)
	(Alcohols)				
1	1-Penten-3-ol	4.3±0.9	13.0±4.4	6.6±2.0	19.7±4.4
2	3-Pentanol	0.5±0.2[5!]	1.9±0.7	0.6±0.3[4]	16.3±4.4
3	1-Pentanol	–	–	–	0.6±0.2
4	(*Z*)-2-Penten-1-ol	–	–	–	1.4+0.7
5	(*Z*)-3-Hexen-1-ol	0.9±0.2	4.1±2.4	1.3±0.5	8.0±3.6
6	1-Hexanol	0.12±0.08[2]	0.04±0.04[1]	0.5±0.2[4]	1.8±1.3
7	3-Heptanol	–	0.04±0.04[1]	–	2.3±0.5
	(Esters)				
	Butyl acetate	1.1±0.2	0.8±0.2	0.7±0.1	0.5±0.2
8	3-Methyl-1-butyl acetate	–	–	–	0.2±0.1
9	(*Z*)-2-Penten-1-yl acetate	0.23±0.06	1.5±0.7[5]	0.4±0.1	12.1±4.0
	(*Z*)-3-Hexen-1-yl acetate	18.2±6.1	38.2±17.7	21.2±11.4	37.8±7.0
10	Hexyl acetate	1.0±0.4[5]	2.5±1.8[5]	0.4±0.2[4]	7.4±3.6
11	(*Z*)-3-Hexen-1-yl-pronanoate	–	–	–	0.4±0.3
	Heptyl acetate	0.06±0.04[2]	–	0.03±0.03[1]	0.3±0.2[4]
12	2-Ethylhexyl acetate	0.5±0.2[4]	0.7±0.3[4]	0.7±0.3[4]	3.7±0.5
	Methyl salicylate	0.03±0.02[2]	0.02±0.02[1]	0.02±0.02[1]	7.2±6.9[4]
	(Nitrogen containing compounds)				
	Methyl thiocyanate	1.6±0.8	0.8±0.1	0.9±0.3	0.4±0.1
13	3-Methylbutanenitrile	0.05±0.05[1]	–	2.0±1.0[4]	4.7±3.6
14	3-Methyl-3-butenenitrile	0.5±0.1[5]	0.8±0.3[4]	0.9±0.2	–
15	Prop-2-enyl isothiocyanate	0.3±0.2[2]	0.2±0.1[5]	0.8±0.3[4]	–
16	But-3-enyl isothiocyanate*	1.8±1.1[5]	0.5±0.4[2]	2.5±0.2	–
	(Ketones)				
17	3-Pentanone	3.5±1.3	11.0±4.1	7.5±2.6	30.0±4.9
18	3-Hexanone	–	–	–	3.5±0.8
19	2-Hexanone	0.41±0.06	0.6±0.1	1.6±1.0	13.8±3.1
20	3-Heptanone	0.58±0.09	1.0±0.2	1.2±0.5	7.9±1.8
21	2-Heptanone	0.17±0.04[5]	0.30±0.06[5]	0.4±0.2	1.6±0.3
	(Sulfides)				
	Dimethyldisulfide	1.7±0.2	2.5±0.9	1.8±0.3	2.4±1.3
	Dimethyltrisulfide	0.49±0.07	0.6±0.1	0.52±0.09	0.5±0.2
	(Terpenes)				
22	α-Thujene*	18.7±3.6	2.7±1.0[5]	14.3±3.8	29.8±8.0
23	α –Pinene*	11.2±2.6	2.5±0.5	8.5±2.1	12.0±3.2
	Thuja-2-4(10)diene	0.05±0.02[5]	0.09±0.02	0.06±0.02[4]	0.08±0.02
24	Sabinene*	54.6±9.0	9.6±3.5	50.4±13.3	49.2±6.2
25	2-β-Pinene*	5.6±1.3	1.2±0.3	4.5±1.1	9.8±3.5
26	β-Myrcene*	14.8±3.2	2.6±0.9[5]	11.0±2.7	25.6±7.3
27	l-Phellandrene	0.03±0.02[2]	0.2±0.2[1]	–	0.4±0.2
28	δ-3-Carene*	0.09±0.04	0.11±0.02	0.4±0.1	0.2±0.1
29	α-Terpinene	0.17±0.06	0.02±0.01[2]	0.09±0.03	1.1±0.6
30	Limonene	24.5±6.1	5.9±1.7	25.1±6.6	27.3±2.8
31	1,8-Cineole*	8.1±2.1	0.7±0.3	4.5±1.1	14.2±4.3
32	Unknown terpenoid*	0.04±0.02[4]	–	0.01±0.01[1]	0.2±0.1
33	(*E*)-β-Ocimene*	0.32±0.07	0.04±0.03[2]	0.09±0.04[3]	1.0±0.8

Table 1 (continued)

ID[b]	Population → Compound names↓	OH (*N*=6)	KIM (*N*=6)	WIN (*N*=5)	CYR (*N*=5)
34	γ-Terpinene*	0.35±0.10	0.06±0.03[3]	0.20±0.08[4]	2.4±1.3
35	Trans-sabinene hydrate*	0.42±0.09	0.04±0.02[2]	0.3±0.1	2.2±1.4
36	α-Terpinonelene*	0.26±0.06	0.04±0.01[5]	0.20±0.06	1.2±0.6
37	*cis*-Sabinene-hydrate	0.03±0.03[1]	–	0.03±03[1]	0.5±0.3
38	DMNT[c]	3.0±0.9	2.3±2.0[3]	0.9±0.6[2]	1.1±0.8
39	Terpinen-4-ol	–	–	–	0.2±0.1[4]
40	α-Terpineol	0.12±0.07[3]	0.01±0.01[1]	0.02±0.02[1]	0.2±0.1
	(*E,E*)-α-Farnesene	0.15±0.07[4]	0.25±0.05	0.38±0.02[3]	0.02±0.02[1]
41	Terpenes*	142±28	28.2±9.4	121±31	178±39
42	Alcohols	5.9±1.3	19.1±7.4	9.0±2.8	50.1±13.7
43	Esters	21.2±6.8	44.0±20.1	24.0±11.6	71.3±16.9
44	N-containing compounds*	4.1±1.3	2.3±0.5	7.1±1.1	5.1±3.7
45	Ketones	4.5±1.3	12.6±4.1	10.3±3.8	55.2±9.2
	Sulfides	2.1±0.3	3.1±1.0	2.4±0.4	2.9±1.5
	Total	180±34	109±40	174±46	363±71

[a] Volatile emissions are given as mean peak area ± SE/g fresh weight of foliage with the number of samples between brackets

[b] ID corresponds with the numbers presented in Fig. 5, 6 and 7

[c] DMNT = (*E*)-3,4,8-dimethyl-1,3,7-nonatriene

!Numbers in superscript following emission quantities give the number of samples in which the compound was detected, if it was not found in all the samples of that population

* Compounds with asterisks indicate significant differences in emission quantities among the three wild populations (*Kruskal-Wallis* one-way ANOVA)

Fig. 5. A PLS-DA performed on WIN and OH samples, which were the least and most attractive populations, respectively, in the flight bioassay, resulted in a model with one significant PC (Fig. 6). From the 16 compounds that were included in the analysis, 3-methyl-butanenitrile and δ-3-carene (both produced in higher amounts by WIN plants, *Mann–Whitney U* tests, $P=0.052$ and $P<0.05$, respectively) and (*E*)-β-ocimene and (*E*)-3,4,8-dimethyl-1,3,7-nonatriene (=DMNT) (both produced in slightly higher amounts by OH plants, *Mann–Whitney U* tests, both $P=0.052$) contributed the most to the separation of the odor blends. Emission of the other 12 compounds did not differ significantly between the two populations. Similarly, CYR and OH volatiles were compared resulting in a model with two significant PC's (Fig. 7). Emissions of the 26 compounds that were included in the model differed significantly (all *P* values≤0.05 *Mann–Whitney U* tests) between OH and CYR plants. The only two compounds that contributed to the separation of the samples and were produced in higher amounts by OH plants were the N-containing volatiles, 3-methyl-3-butenenitrile and but-3-enyl isothiocyanate. However, when OH and WIN plants were compared, quantities of these compounds tended to be higher in WIN than in OH plants (*Mann–Whitney U* test: 3-methyl-3-butenenitrile, $P=0.052$; but-3-enyl isothiocyanate and $P=0.13$). Quantities of (*E*)-β-ocimene and DMNT, which matched the parasitoid preference ranking OH>WIN, did not significantly differ between OH and CYR plants ($P=0.79$ and $P=0.25$, respectively). Thus, emissions of these four compounds do not reflect parasitoid preference.

Discussion

This study showed that there were significant differences between the volatile profiles of the wild *B. oleracea* populations and the cultivar, as well as among the wild populations of this plant. Furthermore, the parasitoid *C. rubecula* was differentially attracted to *B. oleracea* plants from three different wild populations of this species even though they grow less than 20 km from each other in nature. *Cotesia rubecula* also was more attracted to OH plants than to the Brussels sprout variety on which the parasitoid had been reared for many generations. However, *C. rubecula* did not discriminate between volatile blends emitted by the three wild *B. oleracea* populations when treated with JA, a plant hormone that is the key component produced in the lipoxygenase pathway, and which is often used to mimic insect herbivory (Dicke et al. 1999; Thaler 1999; Bruinsma

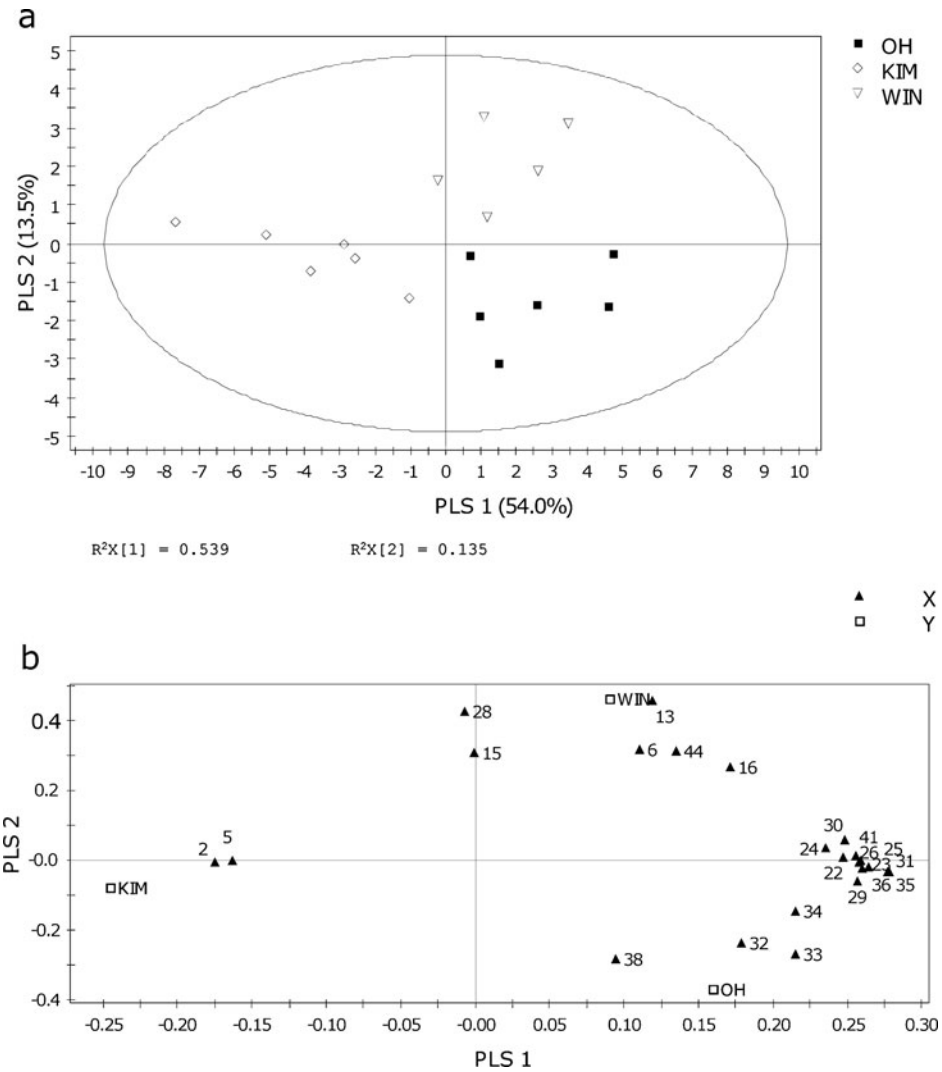


Fig. 5 PLS-DA (Projection to Latent Structures Discriminant Analysis) of quantities of volatile compounds produced by wild *Brassica oleracea* plants originating from three wild populations (OH, KIM, and WIN) in Dorset, UK, in response to *Pieris rapae* feeding. The score plot **a** visualizes the structure of the samples according to the first two PLS components with the explained variance in brackets. The ellipse in the score plot defines the Hotelling's T^2 confidence region, which is a multivariate generalization of Student's *t*-test and provides a 95% confidence interval for the observations. The loading plot **b** define the orientation of the PLS planes with the original variables in the X (volatile variables) and Y space ('class' variables), respectively. They reveal the magnitude and direction of correlation of the original variables with the first two PLS components. The PLS-DA resulted in a model with 3 significant components: R^2X= 0.77, R^2Y=0.82 Q^2=0.67. For compound identity see Table 1

et al. 2009; Zhang et al. 2009). Although each population produced distinctive profiles of volatile chemicals, these did not simply reflect the differences among populations in attractiveness to *C. rubecula.* Moreover, no single chemical matched the differences in attractiveness.

The importance of specific HIPVs in natural enemy attraction traditionally has focused on terpenes, fatty acid derivatives (especially those produced by the lipoxygenase pathway), and products from the phenylalanine lyase (PAL) pathway (Ozawa et al. 2000; Schnee et al. 2006; Shiojiri et al. 2006a; Halitschke et al. 2008; Zhang et al. 2009). Here, we showed that the terpenes (*E*)-β-ocimene and DMNT may play a limited role in *C. rubecula* attraction. For example, exposure to (*E*)-β-ocimene elicits a response in the antennae of *C. rubecula* (Smid et al. 2002) and (*E*)-β-ocimene and DMNT also are produced by Brussels sprout plants in response to exogenous treatment with JA (Bruinsma et al. 2009). Nevertheless, here, *C. rubecula* did not discriminate between JA-treated wild cabbage plants, suggesting that compounds independent of the lipoxygenase pathway are responsible for the differentiation in volatile emissions and consequent attraction of the parasitoid.

Breakdown products of GS represent another important group of volatiles that could be used by parasitoids to detect hosts that are specialist herbivores of brassicaceous plants. Species in this plant family all biosynthesize GS (Halkier and Gershenzon 2006). Glucosinolates function as direct defensive compounds against a range of attackers that include insect herbivores (Rask et al. 2000; Hopkins et al. 2009). Deterrence activity only emerges after tissue rupture, e.g., by caterpillar feeding, and concomitant release of the enzyme myrosinase, which is stored in specialized cells. This enzyme catalyzes the conversion of GS into toxic hydrolysis products of which many are volatile (Halkier and Gershenzon 2006). Glucosinolate concentrations are generally much lower in cultivated brassicaceous plant species compared to the levels found in wild conspecifics because of the effects of directional selection in plant breeding programs (Gols et al. 2008b; Harvey et al. 2011).

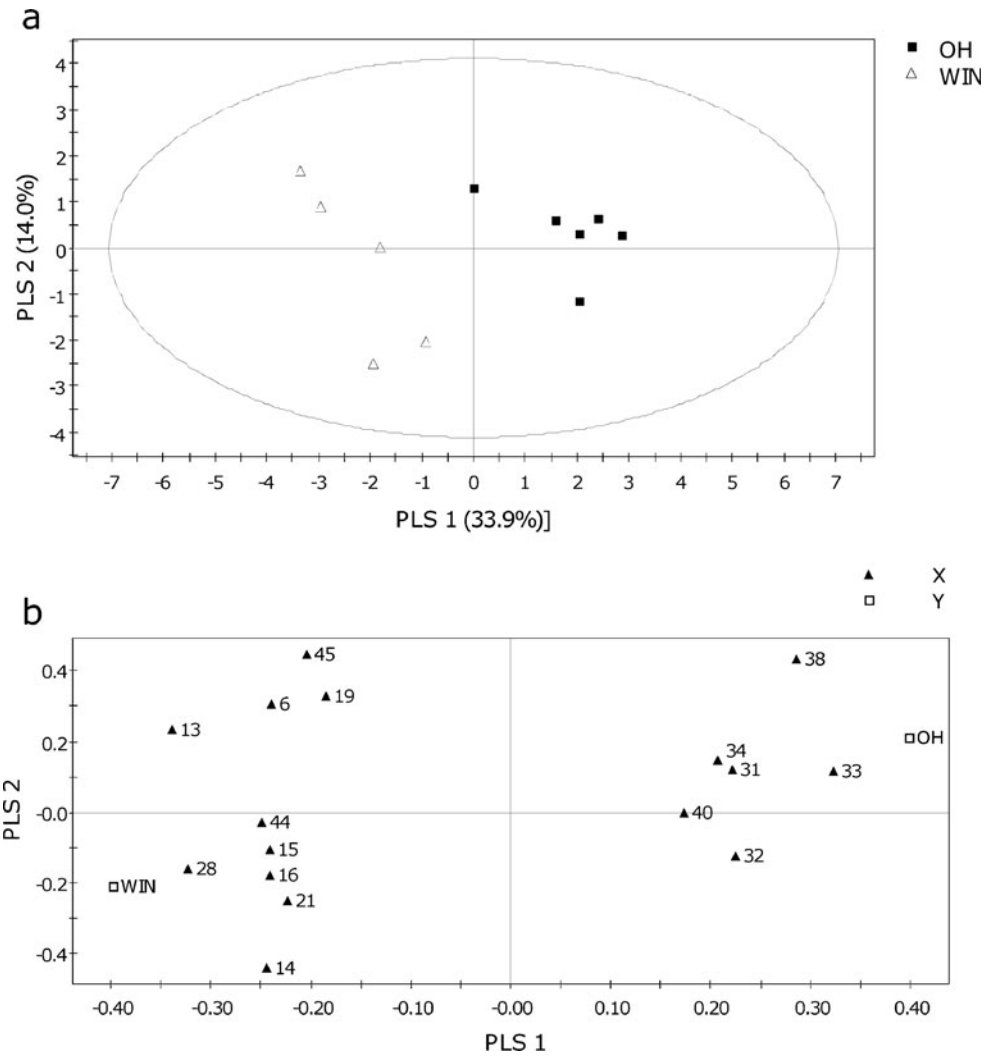


Fig. 6 PLS-DA comparison of quantities of volatile compounds produced by the wild OH and WIN populations of *Brassica oleracea* with the score plot of the samples (**a**) and loadings of the volatile variables (**b**). Percentage of the explained variance is given in brackets. The PLS-DA resulted in a model with 1 significant component: $R^2X=0.34$, $R^2Y=0.83$ $Q^2=0.70$ (the second axis is shown for representational purposes). For compound identity see Table 1

Importantly, the effects of volatile GS breakdown products on parasitoid attraction frequently have been overlooked because these products are not detected in HIPV blends emitted by cultivated brassicaceous plants (Geervliet et al. 1997; Bukovinszky et al. 2005), whereas they are emitted in large quantities by wild species in this family following herbivory (Gols et al. 2008c, 2009). Here, we also found that volatile GS breakdown products, i.e., but-3-enyl isothiocyanate and prop-2-enyl isothiocyanate, were produced only by wild cabbage plants. Thus far, few studies have investigated the attractiveness of GS hydrolysis products to parasitoids (Bradburne and Mithen 2000; Blande et al. 2007). The parasitoid *Diaeretiella rapae*, which attacks the *Brassica*-feeding aphid *Brevicoryne brassicae*, is not only attracted to plants producing isothiocyanates, but female wasps of this species even discriminate between plants producing different isothiocyanates (Bradburne and Mithen 2000). Interestingly, KIM plants had the lowest emission rates of these compounds, suggesting that other cues determine the relative attractiveness to *C. rubecula* of plants from the wild populations.

Volatile emissions are known to vary considerably in quality and quantity, both within and among brassicaceous plant species (Bukovinszky et al. 2005; Gols et al. 2008c). Here, we demonstrated that the volatile blend emitted by KIM plants differed clearly from those emitted by WIN and OH plants, whereas KIM plants were of intermediate attractiveness to *C. rubecula* in the flight bioassay. Our result suggests that the response of *C. rubecula* is based on far more complex choice rules than simple responses to a subset of specific volatile compounds. Several studies have reported that insect natural enemies are able to detect modifications in the emission of terpenes and fatty acid derivatives (Kappers et al. 2005; Shiojiri et al. 2006b; Halitschke et al. 2008). In addition, the emission of repellent compounds may decrease the attractiveness of a volatile blend (e.g., Soler et al. 2007). Combinations of qualitative and quantitative characteristics of the volatile blend could determine the relative importance of specific volatiles in an HIPV blend. Moreover, foraging choices by a parasitoid like *C. rubecula* may not be rigid. For example, pre-adult learning may influence the 'naïve' response of

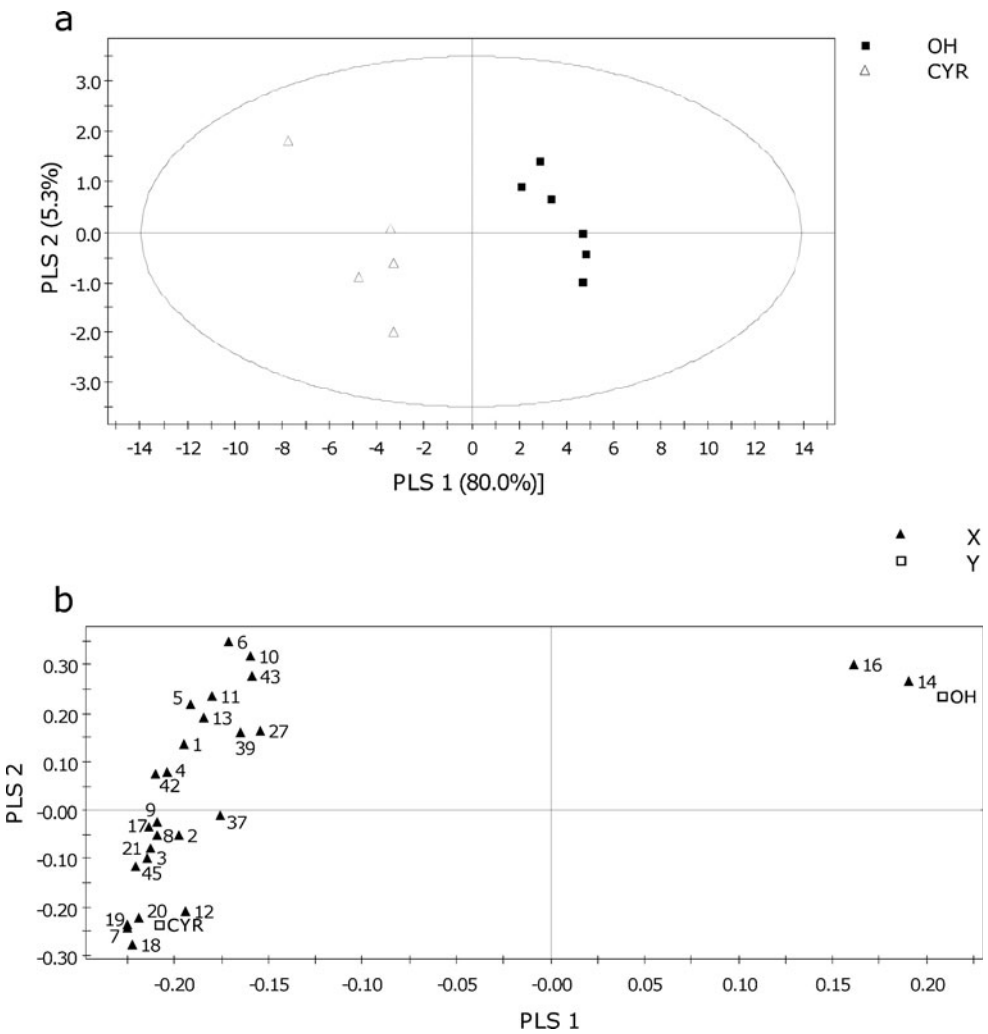


Fig. 7 PLS-DA comparison of quantities of volatile compounds produced by the wild OH population and the CYR cultivar of *Brassica oleracea* with the score plot of the samples (**a**) and loadings of the volatile variables (**b**). Percentage of the explained variance is given in brackets. The PLS-DA resulted in a model with 2 significant components: $R^2X=0.85$, $R^2Y=0.97$ $Q^2=0.90$. For compound identity see Table 1

adult wasps (Gandolfi et al. 2003). Remarkably, *C. rubecula* preferred wild OH over cultivated CYR plants despite their rearing history on the latter plant strain. This result suggests that the naïve response to volatiles emitted by wild plants is conserved. For example, isothiocyanates which were only produced by the wild cabbage plants could be the key components in separating wild from cultivated cabbage; other compounds such as terpenes may facilitate differential attraction among the wild populations.

In nature, *C. rubecula* has to search for hosts that can occur on different plant species in the Brassicaceae. *Pieris rapae* has two to three generations across much of its natural range in central Europe, and most of its food plants are annuals or biennials with short growing seasons. Many of these plants exhibit discrete periods of growth, with some species only occurring early in spring, others in late spring, and still others in summer (Fig. 2). This means that different generations of *C. rubecula* are likely to develop in hosts feeding on different food plant species that emit dramatically different HIPV blends (Bukovinszky et al. 2005; Gols et al. 2008c). Almost all studies of plant-herbivore-parasitoid interactions associated with *B. oleracea* have only used cultivars (Gols and Harvey 2009). Cues, such as volatile GS breakdown products, which are phylogenetically conserved, may be more reliable for naïve parasitoids than more general plant related cues (terpenes, fatty acid derivatives) (Dudareva et al. 2004). Explaining how parasitoids use local information in finding hosts over different spatial scales in natural environments has been little studied. Our findings suggest that it will be worthwhile to investigate how differences in HIPV blends among populations of wild *Brassica* species may structure local host-parasitoid communities, and how artificial selection by domestication impacts these processes.

To summarize, we have shown that there is considerable within and between population variability in volatile profiles of wild and cultivated cabbage plants. Moreover, *C. rubecula* females discriminated among three wild *B. oleracea* populations, as well as between one of the wild populations and a cabbage cultivar on which the parasitoid has been reared for many generations. In parasitoid species whose hosts may feed on different food plants,

selection may favor a flexible response to plant infochemicals when the variation among the blends emitted by different potential food-plant species is high. Although in laboratory studies it has been demonstrated that parasitoids like *C. rubecula* can discriminate between blends that differ in the emission of a single or a few compounds, these results are not conclusive in determining which specific volatiles are important for host plant location in nature. Moreover, the focus of these studies is phytocentric, i.e., unraveling the mechanisms underlying the biosynthesis of these compounds *in planta,* and conclusions are often based on results using cultivated plant species where there is little within-strain variation in the expression of traits like direct and indirect defense (van der Meijden and Klinkhamer 2000; Allison and Hare 2009; Kessler and Heil 2011). As shown here, HIPV blends emitted by cultivated plants whose chemistry has been modified in plant breeding programs may not resemble the volatile blends that are emitted by wild plant species with which the parasitoid has coevolved. Most importantly, these studies to a large extent ignore the biology and ecology of co-evolved multitrophic interactions involving wild plants, herbivores and parasitoids.

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Responses of Bark Beetle-Associated Bacteria to Host Monoterpenes and Their Relationship to Insect Life Histories

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Abstract Bark beetles that colonize living conifers and their microbial associates encounter constitutive and induced chemical defenses of their host. Monoterpene hydrocarbons comprise a major component of these allelochemicals, and many are antibiotic to insects, fungi, and bacteria. Some bark beetle species exhaust these defenses by killing their host through mass attacks mediated by aggregation pheromones. Others lack adult aggregation pheromones and do not engage in pheromone-mediated mass attacks, but rather have the ability to complete development within live hosts. In the former species, the larvae develop in tissue largely depleted of host terpenes, whereas in the latter exposure to these compounds persists throughout development. A substantial literature exists on how monoterpenes affect bark beetles and their associated fungi, but little is known of how they affect bacteria, which in turn can influence beetle performance in various manners. We tested several bacteria from two bark beetle species for their ability to grow in the presence of a diversity of host monoterpenes. Bacteria were isolated from the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, which typically kills trees during colonization, and the red turpentine beetle, *Dendroctonus valens* LeConte, which often lives in their host without causing mortality. Bacteria from *D. ponderosae* were gram-positive Actinobacteria and Bacilli; one yeast also was tested. Bacteria from *D. valens* were Actinobacteria, Bacilli, and γ-Proteobacteria. Bacteria from *D. valens* were more tolerant of monoterpenes than were those from *D. ponderosae*. Bacteria from *D. ponderosae* did not grow in the presence of α-pinene and 3-carene, and grew in, but were inhibited by, β-pinene and β-phellandrene. Limonene and myrcene had little inhibitory effect on bacteria from either beetle species. Tolerance to these antibiotic compounds appears to have resulted from adaptation to living in a terpene-rich environment.

Key Words Actinobacteria · Bacillales · Bacteria · *Dendroctonus ponderosae* · *Dendroctonus valens* · Host defense · Monoterpenes · Polymerase chain reaction (PCR) · γ-Proteobacteria

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Introduction

Bark beetles are major sources of conifer mortality, in scales ranging from localized pockets (Klepzig et al., 1991) to entire landscapes (Aukema et al., 2006; Bentz et al., 2010). Individual trees within a beetle's host range and preferred size class can often resist attack by utilizing terpenoid-based defenses (Lewinsohn et al., 1991; Brignolas et al., 1995; Keeling and Bohlmann, 2006). In particular, monoterpenes can kill or repel bark beetles, and inhibit growth of their fungal symbionts (Smith, 1963; Raffa and Smalley, 1995; Wallin and Raffa, 2000). These compounds are present in

constitutive resin and undergo enhanced production in response to biotic injury, and to a lesser extent to mechanical wounds (Raffa and Berryman, 1983; Raffa and Smalley, 1995). These terpene-based defenses play an important role in constraining populations of eruptive species from transitioning from endemic to epidemic population densities (Boone et al., 2011).

The largest insect outbreak in recorded history currently is underway in western Canada and the U.S. Rocky Mountains (Bentz et al., 2009). Here the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, is causing high rates of mortality to lodgepole pine, *Pinus contorta* var. *latifolia* Dougl., including vigorously growing and well-defended trees (Boone et al., 2011). Elsewhere, they also kill large numbers of ponderosa pines, *Pinus ponderosa* Dougl. Ex Laws, and several other species, including high elevation pines like whitebark pine, *Pinus albicaulis* Engelmann (Logan et al., 2010). Once outbreaks have exhausted preferred hosts, *D. ponderosae* also may attack and kill spruce trees (Furniss and Schenk, 1969; Huber et al., 2009). The ability of these beetles to coordinate pheromone-mediated mass attack allows them to exhaust the resistance of vigorous, well-defended trees, and thereby provide their brood with a substrate with relatively low allelochemical concentrations. The resin content declines rapidly during successful mass attacks (Raffa et al., 1993). These mass attacks typically kill the tree, or in some cases result in strips of dead tissue (Rasmussen, 1974).

Recent warming trends are facilitating range expansion by populations of *D. ponderosae* into new regions and hosts (Carroll et al., 2003). On the east side of the Rocky Mountains in Alberta, mature stands of *P. contorta* are sympatric and hybridize with jack pine, *Pinus banksiana* Lamb. (Critchfield, 1985). Historically, *P. banksiana* has not been exposed to this insect due to the physical barrier of the Rocky Mountains and the cold climate in this region. In 2001, *D. ponderosae* breached this barrier and colonized *P. contorta*, *P. contorta*-*P. banksiana* hybrid, and later *P. banksiana* pines (Nealis and Peter, 2008; Cullingham et al., 2011). The extent to which *D. ponderosae* will impact this region and subsequently the boreal and eastern forests of North America is unknown (Carroll et al., 2003; Logan et al., 2003; Bentz et al., 2010; Safranyik et al., 2010). Similarly, *P. albicaulis* to a large extent historically escaped exposure to sustained *D. ponderosae* populations due to low temperatures of its subalpine habitat, but current warming trends are now facilitating continuous mortality by *D. ponderosae* (Bentz et al., 2010; Logan et al., 2010).

Some stem-colonizing bark beetle species do not engage in pheromone-mediated mass attacks or cause tree death, but rather have the ability to develop within living hosts without killing them (Coulson and Witter, 1984; Berryman, 1986). For example, the black turpentine beetle, *Dendroctonus terebrans* (Oliver) shows evidence of a mating, but not an aggregation, pheromone (Phillips et al., 1990). The red turpentine beetle, *Dendroctonus valens* LeConte, is native to much of North America, and colonizes a large number of pine species (Kelley and Farrell, 1998), and to a lesser extent *Abies*, *Larix*, and *Picea* (Wood, 1982). In the midwestern United States, its major hosts are red pine, *Pinus resinosa* Ait., and *P. banksiana*. A long-term study demonstrated that in Wisconsin, *D. valens* more commonly attacks living red pines prior to lethal attack by *Ips pini* (Say) than the opposite sequence (Aukema et al., 2010). Trees not attacked by *I. pini* the following year continued to live, and did not die until being attacked eventually by this stem-colonizing herbivore (Aukema et al., 2010). Fifty seven percent of these trees were attacked by only one pair of *D. valens* within a year, and 93% of the trees had four or fewer colonizing pairs. Unlike species that must mass attack and kill their hosts prior to reproducing, these beetles appear capable of surviving high monoterpene concentrations in living hosts throughout their development. This is evidenced by descriptions of *D. valens* that complete development in live trees without killing them, and the larvae remain in contact with large amounts of resin in such hosts (Hopkins, 1909; Smith, 1961; Schmid and Mata, 1991; Randall, 2006). In addition to live trees that are often stressed, and whose colonization can lead to subsequent lethal attack by tree-killing species, *D. valens* also colonize stumps from recently cut trees (Furniss and Carolin, 1977; Aukema et al., 2010; Owen et al., 2010). In the late 1990s, *D. valens* invaded parts of China, where it is attacking a new host, Chinese red pine, *Pinus tabuliformis* Carr., in higher numbers, and has become a primary mortality agent (Yan et al., 2005).

Beetles that colonize a diversity of hosts encounter variable monoterpene environments (Seybold et al., 2006). For example, the volatile fraction of *P. contorta* oleoresin is comprised primarily of β-phellandrene and to a lesser extent β-pinene (Zavarin et al., 1969; Raffa and Berryman, 1982a; Pureswaran et al., 2004). β-Phellandrene is rare or absent in other hosts of *D. ponderosae* (Smith, 2000). *Pinus ponderosa* oleoresin contains a more diverse monoterpene profile with relatively equal proportions of 3-carene and β-pinene, and substantial amounts of α-pinene, myrcene, and limonene (Sturgeon, 1979; Hobson et al., 1993). *Pinus albicaulis* oleoresin contains substantial amounts of 3-carene and myrcene (Smith, 2000). *Pinus banksiana* oleoresin contains primarily α-pinene, β-pinene, and 3-carene (Wallin and Raffa, 1999), whereas *P. resinosa* oleoresin contains primarily α-pinene and β-pinene (Raffa and Smalley, 1995).

Bark beetles are associated with a diversity of micro-organisms that facilitate their ability to exploit the subcortical environment (Whitney, 1982; Paine et al., 1997). Of these, fungi have been most thoroughly studied. Conifer mono-terpenes can strongly inhibit some of these fungi, although

toxicity varies among compounds and some fungi are relatively tolerant (Raffa et al., 1985; Paine and Hanlon, 1994; Klepzig et al., 1996; Hofstetter et al., 2005; DiGuistini et al., 2011). Recent studies have shown that some bacterial taxa are frequently associated with bark beetles (Adams et al., 2010; Hulcr et al., 2011) and fulfill important ecological functions. For example, Actinomycete bacteria associated with two beetle species inhibit the beetles' fungal antagonists (Cardoza et al., 2006; Scott et al., 2008), and Proteobacteria stimulate the growth and reproduction of beetle symbiotic fungi in the presence of the host compound α-pinene (Adams et al., 2009). Bacteria inhabiting guts of other wood-feeding insects, such as termites and cerambycid beetles, degrade plant-cell wall components, such as cellulose and lignin, and contribute to acquisition of nutrients from these generally recalcitrant woody-substrates (Warnecke et al., 2007; Geib et al., 2008). Some bacteria associated with bark beetles tolerate myrcene (Skrodenyte-Arbaciauskiene et al., 2006) and α-pinene (Adams et al., 2009), but their tolerance to other monoterpene components of conifer defense is unknown.

In this study, we tested how bacteria isolated from two bark beetle species that exhibit different host defense tolerances associated with their different host colonization behaviors (i.e., killing trees by mass attack vs. colonizing live trees without mass attacks or causing mortality) are affected by a diversity of monoterpenes from pine oleoresin. Specifically, we isolated bacteria from *D. ponderosae* and *D. valens* and exposed these bacteria to monoterpenes that comprise the major resin and phloem constituents of *P. contorta*, *P. banksiana*, *P. resinosa*, and *P. albicaulis*. We hypothesized that bacteria from both beetle species will grow in the presence of many monoterpenes, and that bacteria associated with *D. valens* will be more capable of tolerating these compounds given its life history.

Methods and Materials

Isolation of Microorganisms *Dendroctonus ponderosae* adults were collected in July 2008 from under the bark of naturally attacked trees in a stand of *P. contorta* and *P. contorta—P. banksiana* hybrid pines, near Grande Prairie, Alberta, Canada (54°43′N, 119°39′W). Beetles of both sexes were actively tunnelling in the egg gallery at the time of collection. *Dendroctonus valens* adults of both sexes were collected in May 2008 from under the bark of naturally colonized *P. resinosa* stumps in a spring-thinned plantation in Black River State Park, Black River Falls, WI, USA (44°14'57 N, 90°34'07"W). These beetles were actively tunnelling in egg galleries as well. For all beetles collected in this study, galleries were clear of resin and appeared to represent successful colonization of the host. During sampling, each adult was placed into individual, sterile vials, placed on ice, and transferred to the lab and stored at 4°C.

Within 24 hr, at least 25 beetles from each species were surface washed in phosphate buffered saline, pH 7.4 (Sigma-Aldrich Co., St. Louis, MO, USA) (PBS) with 1% Tween 20 (ICI America Inc., Wilmington, DE, USA) by vortexing for 30 sec, and rinsed in PBS. Bacteria were isolated by crushing beetles in PBS and plating serially diluted aliquots of the samples on 10% tryptic soy agar (TSA; Difco, Sparks, MD, USA). Petri dishes were stored at room temperature for up to 2 week, and colonies from a diversity of morphologies were selected from multiple beetles and obtained in pure culture for bioassay.

All bacteria were identified by direct sequencing of regions V5 of the rRNA 16S gene. Polymerase chain reaction (PCR) was used to amplify partial ribosomal RNA (rRNA) gene sequences (Holben et al., 2002), with the exception that PCR amplicons were used in direct sequencing reactions rather than for cloning. The primers 27f (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') (based on the *Escherichia coli* numbering system) were used for PCR. PCR reaction mixtures contained 25 µl GoTaq Green Mastermix (Promega, Madison, WI, USA), 2 µl of each primer 20 µl of water, and 1 µl of culture. PCR reactions were performed by using an initial denaturation of 5 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and primer extension at 72°C for 2 min. This was followed by a final extension reaction at 72°C for 10 min. Sequencing of PCR products was performed by the University of Wisconsin Biotechnology Center (Madison, WI, USA). Sequences were deposited in GenBank (Table 1). National Center for Biotechnology Information (NCBI) similarity scores were obtained by using the NCBI search of previously deposited sequences in GenBank (www.ncbi.nlm.nih.gov).

Effects of Monoterpenes on Growth of Bacteria We quantified the effects of monoterpenes on growth of bacteria by using methods described by Middelbeek et al. (1992) and Adams et al. (2009). Briefly, 10 µl of actively growing culture were inoculated into 200 µl of 10% tryptic soy broth in 96-well plates (Becton Dickinson and Co., Franklin Lakes, NJ, USA). Wells were amended with 0%, 1%, or 5% concentrations (*v*/*v*) of one of 6 monoterpenes for isolates from *D. ponderosae*, and 5 monoterpenes for isolates from *D. valens*. The monoterpenes were racemic α-pinene (98%, Aldrich, Milwaukee, WI, USA), (−)-β-pinene (technical grade, approx. 86%, Aldrich), (*R*)-(+)-limonene (98%, Aldrich), myrcene (tech. grade, approx. 78%, Aldrich), and (+)-3-carene (90%, Aldrich), which were tested against all isolates, and β-phellandrene (Glidco

Table 1 GenBank accession numbers for bacteria isolated from *Dendroctonus ponderosae* and *D. valens*

Isolate source	Isolate name	Class of closest match in GenBank	Closest named match in GenBank (accession #)	Sequence length (bp)	Percent similarity	Accession No. of isolate
D. ponderosae	A7-1a	Actinobacteria	*Micrococcus terreus* (HQ009859)	1216	99	HQ728081
	A3-1a	Bacilli	*Paenibacillus xylanexedens* (HM439462)	758	100	HQ728079
	A5-1	Bacilli	*Paenibacillus* sp. (GQ920787)	383	99	HQ728082
	A3-1b	Bacilli	*Bacillus simplex* (FJ544333)	551	98	HQ728080
	A9-4[a]	Saccharomycetes	*Candida piceae* (EU011713)	526	100	HQ728083
D. valens	Dv3	γ-Proteobacteria	*Enterobacter* sp. (AY082447)	1320	98	HQ728086
	Dv6	γ-Proteobacteria	*Enterobacter amnigenus* (DQ481471)	1320	100	HQ728087
	Dv25	γ-Proteobacteria	*Pseudomonas* sp. (AM913961)	942	99	HQ728088
	Dv26	Actinobacteria	*Frigoribacterium* sp. (AF157479)	1350	100	HQ728084
	Dv34	Bacilli	*Paenibacillus provencensis* (EF212893)	849	100	HQ728085

[a] Fungus

Organics Corp., Jackson, MS, USA), which was tested against isolates from *D. ponderosae*. Individual wells were sealed by using VIEWseal pressure sensitive adhesive (Greiner Bio-one, Maybachstraße, Germany). Cultures were shaken at medium speed (500 revolutions per min) and orbital directionality at 24°C, and optical density was measured every hour for up to 48 h by using a DXT 880 Multimode Detector (Beckman Coulter, Corona, CA, USA) at absorbance of 595 nm. Each isolate was exposed to each monoterpene and tested an average of two times to verify consistent growth patterns (Table 2).

Data Analysis We compared the doubling times of bacteria with and without monoterpene addition during the exponential phase of growth (Middelbeek et al., 1992). To determine the time period in which the exponential phase took place, growth of bacteria were monitored for up to 48 h, a period that encompassed the growth phase. Next, absorbance values were log transformed and the time interval with the most linear log-transformed absorbance values was selected by calculating the r^2 values of each possible time interval (StatView version 4.57, Abacus Concepts, Inc., Berkeley, CA, USA). During this period of exponential growth, an index of monoterpene impact on bacterial growth rate was calculated by using methods described by Middelbeek et al. (1992): the difference in absorbance values at the beginning and end of the exponential growth phase (representing the number of cell divisions) was divided by the $\log_2$ of the duration (number of hours) of the exponential growth phase. The reciprocal of this value is then taken to provide the number of hours per generation, or doubling time. The doubling time of each bacterium growing in each terpene concentration was calculated relative to controls by subtracting the doubling time of the isolate growing without terpene from the doubling time of the isolate growing with the terpene, and dividing this value by the doubling time of the isolate growing without terpene, x 100. Relative doubling times were grouped into five categories: −75% to −25%, stimulated growth; −25% to 25%, growth not affected; 25% to 100%, growth inhibited; > 100%, growth strongly inhibited; and no growth, complete inhibition.

Results

Isolation and Identification of Microorganisms Bacteria closely related to gram-positive actinobacterium *Micrococcus terreus* (isolate A7-1a), and Bacillales *Paenibacillus* spp. (isolates A3-1a and A5-1) and *Bacillus simplex* (isolate A3-1b) were isolated from *D. ponderosae* and tested in this study (Table 1). One yeast isolate (A9-4) most closely related to *Candida piceae*, also was obtained in culture and used in bioassays (Table 1).

Bacteria most closely related to an actinobacterium *Frigoribacterium* sp. (isolate Dv26) and Bacillales *Paenibacillus provencensis* (isolate Dv34) were isolated from *D. valens* and tested (Table 1). Three other isolates were most closely related to gram-negative γ-Proteobacteria *Enterobacter* spp. (isolates Dv3 and Dv6) and *Pseudomonas* sp. (isolate Dv25) (Table 1).

Effects of Monoterpenes on Growth Monoterpenes had substantial effects on bacterial growth (doubling time), and these varied with compound, isolate, and insect host (Table 2). Overall, growth of bacteria was most inhibited by (+)-3-carene and (±)-α-pinene, and was least inhibited by myrcene and limonene. Six of the ten isolates grew in the presence of α-pinene (Table 2, Fig. 1). The four isolates that

Table 2 Doubling time (h) of microbial isolates from *Dendroctonus ponderosae* (MPB) and *D. valens* (RTB) adults in the presence and absence of monoterpenes

Doubling time ± 90% C.I. (*N*)

Source	isolate	Conc. (%)	α-Pinene	β-Pinene	Limonene	Myrcene	3-Carene	β-Phellandrene
MPB	A7-1a	0	4.7±0.03 (2)	8.8±0.71 (4)	5.2±0.63 (4)	5.5±0.27 (2)	6.9±0.56 (4)	6.9±0.73 (3)
		1	11.1±0.48 (4)	12.7±0.24 (3)	8.7±2.54 (3)	4.0±0.01 (2)	CI (4)	6.6±0.51 (3)
		5	13.3±1.67 (2)	40.4±11.31 (4)	8.3±2.90 (3)	4.4±0.25 (2)	CI (4)	9.4±0.64 (3)
	A3-1a	0	5.3±0.07 (2)	6.6±0.09 (4)	5.0±0.16 (2)	3.6±0.10 (2)	4.9±0.49 (3)	14.9±2.37 (4)
		1	CI (2)	31.3±3.11 (2)	3.5±0.12 (2)	3.8±0.34 (2)	CI (3)	20.0±1.80 (3)
		5	CI (2)	20.6±1.13 (2)	3.1±0.03 (2)	2.8±0.25 (2)	CI (3)	21.7±3.52 (4)
	A3-1b	0	5.1±0.18 (2)	6.7±0.01 (3)	7.2±1.00 (4)	15.5±0.01 (2)	10.3±0.17 (2)	13.4±0.50 (2)
		1	CI (2)	9.1±0.02 (3)	8.1±0.19 (3)	17.7±0.45 (2)	17.8±0.06 (2)	17.5±1.81 (2)
		5	CI (2)	CI (3)	7.8±1.03 (3)	16.3±0.71 (2)	CI (2)	19.4 (1)
	A5-1	0	4.7±0.08 (2)	6.7±0.28 (2)	4.8±0.11 (2)	5.3±0.25 (2)	8.2±0.11 (2)	9.9±0.48 (4)
		1	CI (2)	10.3±0.37 (2)	4.9±0.01 (2)	5.3±0.20 (2)	CI (2)	17.4±2.10 (2)
		5	CI (2)	10.5±0.45 (2)	5.1±0.03 (2)	7.6±0.56 (2)	CI (2)	14.4±1.18 (2)
	A9-4	0	3.9±0.02 (2)	8.4±0.05 (2)	5.6±0.52 (4)	11.0±0.48 (2)	10.5±0.04 (2)	9.4±0.62 (4)
		1	CI (2)	8.2±0.69 (2)	6.4±0.42 (3)	4.7±0.23 (2)	13.2±0.71 (2)	13.3±0.91 (4)
		5	CI (2)	7.6±0.38 (2)	7.7±0.53 (2)	7.6±0.64 (2)	CI (2)	16.5±1.75 (3)
RTB	Dv3	0	5.0±0.31 (2)	3.3±0.12 (2)	no data	3.1±0.12 (2)	4.1±0.02 (2)	
		1	6.6±0.80 (2)	5.0±1.17 (2)	3.3 (1)	3.3±0.80 (2)	3.7±0.20 (2)	
		5	26.4±4.45 (2)	3.1±0.43 (2)	3.3±0.24 (2)	2.5±0.09 (2)	8.9±1.02 (2)	
	Dv6	0	4.1±0.02 (2)	4.2±0.08 (2)	3.4±0.17 (2)	4.0 (1)	4.3±0.05 (2)	
		1	3.5±0.01 (2)	5.4 (1)	3.3±0.34 (2)	3.5 (1)	5.7±0.02 (2)	
		5	4.8±0.00 (2)	4.4±0.38 (2)	3.7±0.42 (2)	4.9±0.22 (2)	5.7±0.31 (2)	
	Dv25	0	3.7±0.09 (2)	3.5±0.03 (2)	4.7±0.01 (2)	3.1±0.12 (2)	4.7±0.04 (2)	
		1	3.8±0.25 (2)	5.4±0.49 (2)	5.1±0.23 (2)	2.6±0.14 (2)	3.9±0.01 (2)	
		5	4.3±0.17 (2)	4.7±0.03 (2)	5.9±0.58 (2)	3.1±0.06 (2)	4.5 (1)	
	Dv26	0	2.9±0.10 (2)	3.0±0.23 (2)	5.1±0.22 (2)	3.5 (1)	3.0±0.14 (2)	
		1	3.3±0.17 (2)	4.4±0.25 (2)	3.6 (1)	3.2 (1)	3.0±0.10 (2)	
		5	4.8±0.19 (2)	3.8±0.00 (2)	8.6±0.52 (2)	3.4±0.26 (2)	4.5 (1)	
	Dv34	0	5.4±0.00 (2)	4.6±0.15 (2)	3.4±0.17 (2)	3.3±0.14 (2)	4.7±0.02 (2)	
		1	5.0±0.23 (2)	5.2±0.13 (2)	3.3±0.34 (2)	3.2±0.47 (2)	4.0±0.28 (2)	
		5	2.9±0.08 (2)	4.0±0.01 (2)	4.0±0.40 (2)	3.8±0.24 (2)	4.6±0.02 (2)	

For table entries labeled “CI”, the isolates were completely inhibited

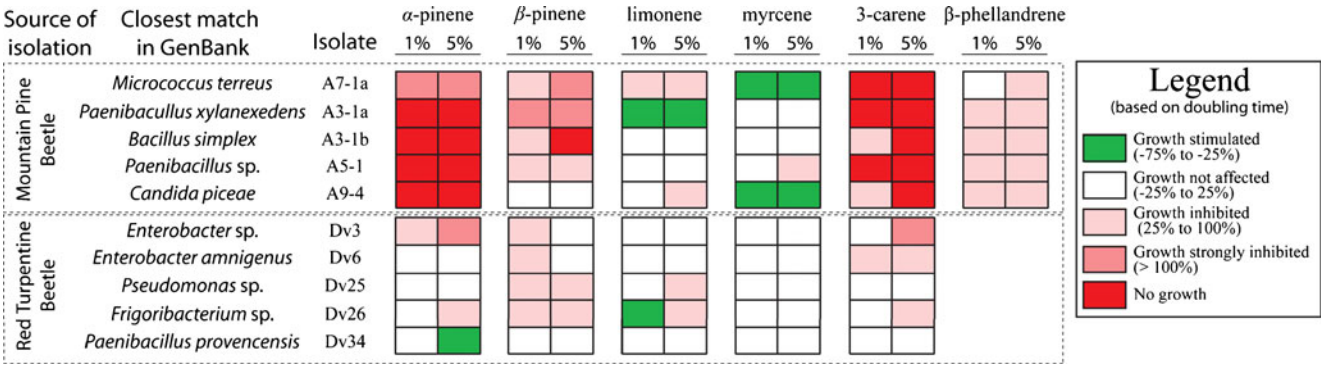


Fig. 1 Heat map representing the growth of bacterial isolates growing in the presence of monoterpenes relative to growing in the absence of monoterpenes. Percentages listed in the legend represent doubling time of each isolate growing with monoterpenes at 1% and 5% concentrations relative to each isolate growing in culture without terpenes

did not grow in any concentration of (±)-α-pinene were all isolated from *D. ponderosae*. One isolate, A7-1a, from *D. ponderosae* grew in, but was strongly inhibited by, both concentrations of α-pinene. All isolates from *D. valens* grew at 1% α-pinene, with no effect on four isolates and inhibition of one, isolate Dv3. All isolates also grew at 5% α-pinene, with an increase in growth of Dv34, no effect on Dv6 and Dv25, inhibition of Dv26, and strong inhibition of Dv3.

All isolates grew in the presence of (*S*)-(−)-β-pinene (Table 2, Fig. 1). All isolates from *D. ponderosae* grew at 1% β-pinene, and all but one isolate, A3-1b, grew at 5% β-pinene. Three isolates, A7-1a, A3-1b, and A5-1, were inhibited and isolate A3-1a was strongly inhibited at 1%. Isolate A5-1 was inhibited and isolates A7-1a and A3-1a were strongly inhibited at 5%. Growth of isolate A9-4 was not affected, and isolate A3-1b did not grow at 5%. All isolates from *D. valens* grew in both concentrations of β-pinene. All but isolate Dv34 were inhibited at 1%. At 5%, three isolates were unaffected, and Dv25 and Dv26 were inhibited.

All isolates grew in the presence of (*R*)-(+)-limonene (Table 2, Fig. 1). Isolate A3-1a from *D. ponderosae* was stimulated at both concentrations, isolate A7-1a was inhibited at both concentrations, and isolate A9-4 was inhibited at 5%. Three isolates from *D. valens* were unaffected by either concentration, and growth of isolate Dv26 was stimulated at 1%, and growth of isolates Dv25 and Dv26 was inhibited at 5%.

All bacterial isolates grew in the presence of myrcene (Table 2, Fig. 1). Isolates A7-1a and A9-4 from *D. ponderosae* were stimulated at both concentrations, and growth of isolates A3-1a and A3-1b was not affected at either concentration. Isolates A3-1a and A3-1b were unaffected at either concentration of myrcene and isolate A5-1 was inhibited at 5%. Growth of all isolates from *D. valens* was unaffected by myrcene.

All but two isolates were inhibited by (+)-3-carene under some condition (Table 2, Fig. 1). Isolates A7-1a, A3-1a, and A5-1 from *D. ponderosae* did not grow at either concentration, and isolates A3-1b and A9-4 were inhibited at 1% and did not grow at 5%. All bacteria from *D. valens* grew at both concentrations of (+)-3-carene. Isolate Dv6 was inhibited at 1% and 5%, and isolate Dv26 was inhibited and isolate Dv3 was strongly inhibited at 5%.

β-Phellandrene inhibited the growth of all bacteria from *D. ponderosae*, and all but isolate A7-1a were inhibited at 1%. However, no bacteria were totally or even strongly inhibited at either concentration (Table 2, Fig. 1).

Discussion

Bacteria isolated from *D. ponderosae* were more strongly inhibited by exposure to monoterpenes than those isolated from *D. valens*. This appears to correspond with a major difference in their life histories, namely that *D. ponderosae* deplete host monoterpene concentrations by mass attack, whereas *D. valens* in Wisconsin often conduct single or low density attacks and complete development within a terpene-rich environment. This pattern occurred even though similar bacterial taxa were tested. For example, Actinobacteria and Bacillales isolates from *D. valens* were more tolerant than those from *D. ponderosae* (Table 1). Actinobacteria occur in associations with both pine phloem (Adams et al., 2008; Hulcr et al., 2011) and bark beetles (Cardoza et al., 2006, 2009; Scott et al., 2008), so some tolerance to monoterpenes would seem necessary for their survival. Some bacteria in the Bacillales are capable of degrading various monoterpenes (Chang and Oriel, 1994; Savithiry et al., 1998) and similar hydrocarbons synthesized by plants (Wright et al., 1986; Jiménez et al., 2010). Unlike the isolates from *D. ponderosae*, three of the isolates from *D. valens* were γ-Proteobacteria.

The tolerance to monoterpenes by the yeast isolate from *D. ponderosae* is not entirely unexpected, given associations of yeasts with bark beetles (Shifrine and Phaff, 1956; Boone et al., 2008b; Davis et al., 2011). The adaptation of yeasts to the terpenoid-rich environment may be particularly beneficial for some species of bark beetles, as yeasts have been proposed to contribute to pheromone synthesis (Brand et al., 1977) and favor advantageous fungi (Davis et al., 2011). However, the extent to which yeasts contribute to pheromone communication in nature is uncertain, and some *Ips*-associated yeasts are relatively sensitive to some terpenes (Leufvén et al., 1988; Seybold et al., 2000). Yeast survival also could adversely affect larvae, as some natural enemies of bark beetles are attracted to yeast metabolites (Boone et al., 2008b).

Bacteria from *D. ponderosae* were more tolerant of β-phellandrene than (±)-α-pinene and (*S*)-(−)-β-pinene. β-Phellandrene is the predominant monoterpene of the primary host of *D. ponderosae*, whereas α- and β-pinene are the predominant monoterpenes of *P. banksiana*, which is the host into whose range populations of *D. ponderosae* are expanding. Further, these bacteria were highly tolerant of, and their growth was stimulated by, myrcene, which *D. ponderosae* exploits as a synergist of its aggregation pheromone to mass attack trees (Miller and Lindgren, 2000; Boone et al., 2008a; Borden et al., 2008). This compound also is an important component of *P. albicaulis* resin (Smith, 2000).

The antibacterial activity of 3-carene is particularly noteworthy. Specifically, this compound is correlated with resistance of conifers to *D. ponderosae* and the white pine weevil, *Pissodes strobi* (Peck) (Coleoptera: Curculionidae) (Ott, 2009; Robert et al., 2010), yet paradoxically frequently proves to be among the least bioactive in insect and fungal assays (Raffa et al., 1985; Klepzig et al., 1996; Lu et al., 2010). The antibacterial activity of 3-carene may be a mechanism that assists in tree resistance. Although 3-carene has a strong antibiotic effect on bacteria isolated from *D. ponderosae*, it caused little inhibition of bacteria isolated from *D. valens*. 3-Carene is a major volatile attractant (kairomone) for the flight response of *D. valens* adults (Hobson et al., 1993; Erbilgin et al., 2007). It is possible that bacteria closely associated with *D. valens* have adapted to be tolerant of this compound, given their inevitable exposure once the beetle penetrates the host.

α-Pinene, which occurs in *P. contorta* at low concentrations (Zavarin et al., 1969; Raffa and Berryman, 1983) but is a more predominant monoterpene in other hosts of *D. ponderosae* such as *P. ponderosa* and *P. albicaulis* (Sturgeon, 1979; Smith, 2000), was strongly antibiotic to microbes from *D. ponderosae*. In another study, however, *D. ponderosae*-associated bacteria tolerated α-pinene (Adams et al., 2009). This distinction may be due to difference among bacterial taxa tested, specifically *Pseudomonas* sp. that are tolerant of a diversity of aromatic compounds (Jiménez et al., 2010). α-Pinene also inhibits growth and germination of fungi associated with *D. valens* (Klepzig et al., 1996).

Two monoterpenes that all microbes tolerated were limonene and myrcene. These monoterpenes are found in relatively low concentrations in volatile profiles of many conifers, with an exception in *P. albicaulis* in which myrcene is a dominant component (Smith, 2000). Myrcene increases the attraction of the aggregation pheromone of *D. ponderosae* (Miller and Lindgren, 2000; Boone et al., 2008a; Borden et al., 2008) but has no or very little significant effect on the flight response of *D. valens* (Hobson et al., 1993; Sun et al., 2004). No evidence exists for a role of limonene in communication in *Dendroctonus*, but it is an attractant of *Ips typographus* (Reddemann and Reinhard, 1996). Myrcene and limonene inhibit growth of several bark beetle-associated fungi that colonize *P. jeffreyi* and *P. contorta* and grand fir, *Abies grandis* (Raffa et al., 1985; Paine and Hanlon, 1994), hosts with low concentrations of myrcene (Raffa and Berryman, 1982a,b, 1983; Smith, 2000). Myrcene also inhibits growth of fungi associated with *D. frontalis* Zimmerman to some extent; however, limonene has little effect on the same fungi (Hofstetter et al., 2005). Further work is necessary to fully characterize the antibiotic effects of limonene and myrcene on bark beetle-associated microorganisms.

Two of the most frequently reported bacteria associated with *Dendroctonus* beetles include *Bacillus* spp. and γ-Proteobacteria such as *Enterobacter* spp. and *Pseudomonas* spp., which have been detected with *D. valens*, *D. ponderosae*, spruce beetle (*D. rufipennis* Kirby), and *D. frontalis* (Cardoza et al., 2009; Morales-Jiménez et al., 2009; Adams et al., 2010). The other bacteria used in this study also have been detected in bark beetles, including *Micrococcus* spp. from *D. rufipennis* and *D. ponderosae* (Adams et al., 2008; Cardoza et al., 2009), and *Paenibacillus* sp. and *Frigoribacterium* sp. from *I. pini* (Delalibera et al., 2007; Cardoza et al., 2009). Yeasts such as *Candida* spp. previously have been isolated from a diversity of bark beetles, including *D. ponderosae* (Adams et al., 2008). Future studies are needed to determine the activities of blends of compounds, better quantify the frequencies of association of various bacterial taxa with bark beetles, better partition geographic ranges and life history strategies, and construct comparisons with outlier groups to improve our evolutionary understanding. With increasing focus on bacteria associated with bark beetles and insects in general, and new tools such as next-generation sequencing, our understanding of these ecological relationships will likely improve.

The chemical defenses of conifers provide strong resistance mechanisms to colonization by biotic agents, as is evident from the relatively few insects and pathogens that

colonize the phloem of living trees. We hypothesized that the ability of bark beetles that colonize living hosts to establish in new environments and hosts, which are facilitated by climate warming trends and human-facilitated transportation (Sun et al., 2004; Yan et al., 2005; Logan et al., 2010), may be in part due to activities of their microbial symbionts. Microbial symbionts perform a variety of functions important to the success of their animal hosts (Zilber-Rosenberg and Rosenberg, 2008), so their ability to survive a harsh environment, such as within trees, likely is critical to their animal host. In this study, we show that microbes associated with two species of bark beetle survived and grew in the presence of antimicrobial components of the chemical defense of conifers. Future studies are needed to investigate the metabolic capabilities of bacteria growing in the presence of individual resin components and blends.

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Silencing Defense Pathways in *Arabidopsis* by Heterologous Gene Sequences from *Brassica oleracea* Enhances the Performance of a Specialist and a Generalist Herbivorous Insect

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Abstract The jasmonic acid (JA) signaling pathway and defensive secondary metabolites such as glucosinolates are generally considered to play central roles in the defense of brassicaceous plants against herbivorous insects. To determine the function of specific plant genes in plant-insect interactions, signaling or biosynthetic mutants are needed. However, mutants are not yet available for brassicaceous plants other than *Arabidopsis thaliana,* e.g., cabbage (*Brassica oleracea*). We employed virus-induced gene silencing (VIGS) by using tobacco rattle virus (TRV) to knock down the endogenous expression of lipoxygenase (*LOX*), an upstream enzyme of the JA pathway and thioglucoside glucohydrolase: myrosinase (*TGG1/TGG2*), a hydrolytic enzyme that catalyzes the release of defensive volatile products originating from glucosinolates, in *Arabidopsis thaliana.* This was done by using the heterologous gene sequences from *B. oleracea.* Silencing these genes in *A. thaliana* plants is efficient and specific. Only 18 nucleotides with 100% identity between the trigger (*BoMYR*) and the target (*AtTGG1/2*) sequence are sufficient to achieve gene silencing. *LOX*-silenced plants showed significantly reduced *AtLOX2* transcript accumulation after *Pieris rapae* larval feeding. *TGG*-silenced plants exhibited significantly lower *TGG1/TGG2* transcript levels only after shorter larval feeding. The inhibition of *TGG1/TGG2* transcript accumulation *via* gene silencing may be overruled by longer larval feeding. Specialist *P. rapae* larvae developed significantly better on both types of silenced plants than on empty vector (EV) control plants, while generalist *Mamestra brassicae* larvae developed significantly better on the *TGG1/TGG2* silenced plants than on EV control plants. This shows that not only the generalist herbivore but also the Brassicaceae-specialist *P. rapae* is negatively affected by the ability of brassicaceous plants to produce their specific secondary metabolites, i.e., glucosinolates. Our results demonstrate the important roles of *AtLOX2* and *AtTGG1/TGG2* genes, which were silenced by heterologous gene sequences from *B. oleracea BoLOX* and *BoMYR,* in *A. thaliana* resistance to the specialist *P. rapae* and the generalist *M. brassicae.*

Key Words *Arabidopsis thaliana* · Lipoxygenase (*LOX*) · Myrosinase (*MYR*) · *Pieris rapae* · *Mamestra brassicae* · Virus-induced gene silencing (VIGS)

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Introduction

An ecological genomics approach may contribute to the enhancement of ecological knowledge of interactions between different species (Kessler and Baldwin, 2002; Zheng and Dicke, 2008). Hence, molecular genetic knowledge and methods should be developed for ecological model species by transferring methods used for molecular genetic model

species to ecological model species. Advanced molecular techniques that were developed for *Arabidopsis thaliana*, e.g., microarrays, have been applied to *Brassica oleracea* for studying insect-induced transcriptional responses (Broekgaarden et al., 2007, 2008; Fatouros et al., 2008). *Brassica oleracea* is a well-studied ecological model species (Mattiacci et al., 1995; Eigenbrode et al., 1996; Shiojiri and Takabayashi, 2003; van Dam and Raaijmakers, 2006; Bukovinszky et al., 2008; Fatouros et al., 2008; Gols et al., 2008). However, thus far, little information at the molecular level has been available for this species.

To address the involvement of plant genes in *Brassica*-insect interactions, mutant plants are currently lacking that are altered in signaling or biosynthetic pathways involved in defense against insects. In contrast, such mutants are abundantly available for *A. thaliana*,. Therefore, other techniques are required to elucidate gene function. Virus-induced gene silencing (VIGS) offers an attractive alternative (Mitra et al., 2008; Pandey and Baldwin, 2008; Mantelin et al., 2009). Initially, VIGS was almost exclusively performed in the model plant species *Nicotiana benthamiana* by using vectors derived from tobacco mosaic virus (TMV) (Kumagai et al., 1995), potato virus X (Ratcliff et al., 1997), and tobacco rattle virus (TRV) (Ratcliff et al., 2001; Liu et al., 2002). In recent years, new protocols and vectors have expanded the list of both monocotyledonous and dicotyledonous plant species in which VIGS can be employed (Robertson, 2004; Scofield and Nelson, 2009). Currently, the most widely used VIGS vectors are based on TRV. Such VIGS vectors have been used to silence genes mainly in a number of solanaceous plant species, including tobacco, tomato, pepper, potato, and petunia (Shao et al., 2008). Optimized procedures aimed at improving the efficiency of agro-infiltration of *A. thaliana* have been reported in recent years (Burch-Smith et al., 2006; Wang et al., 2006; Pflieger et al., 2008; Zheng et al., 2010). VIGS has obvious advantages over other known approaches for gene function analysis (Scofield and Nelson, 2009). It is a rapid method that does not require the development of stable genetic transformation. Moreover, it can be applied for functional analysis of genes for which only partial sequences are available (Shao et al., 2008). This is especially important and useful because characterization of gene function *via* generating mutants by genetic transformation in *Brassica* species is still difficult and time-consuming.

Unfortunately, VIGS is not yet successfully developed for our ecological model plant *Brassica oleracea* and, therefore, we took an intermediate step in using *B. oleracea* gene sequences to silence defense-related genes in *Arabidopsis*. We developed a loss-of-function approach with TRV-mediated VIGS for *A. thaliana* plants (Zheng et al., 2010). We employed a heterologous gene sequence, i.e., *phytoene desaturase* (*BoPDS*) from *B. oleracea,* to silence its ortholog in *A. thaliana* plants. We found that disruption of this specific gene of the carotenoid biosynthetic pathway through VIGS affected vision-mediated oviposition behavior of the butterfly *Pieris rapae* (Zheng et al., 2010). In the present study, we used *B. oleracea* gene sequences to silence two genes, lipoxygenase (*AtLOX*) and thioglucoside glucohydrolase: myrosinase (*AtTGG*) in *A. thaliana* plants because silencing *BoLOX* and *BoMYR* in cabbage plants themselves has not yet been successful.

The *BoLOX* gene is likely to catalyze the first step in the oxylipin pathway leading to jasmonic acid (JA) biosynthesis, which is induced by mechanical wounding and herbivory (Zheng et al., 2007). The transcript levels of *BoLOX* are dramatically increased upon infestation by different herbivores and a pathogen (Zheng et al., 2007). However, it is still not clear whether *BoLOX* has the same function as *AtLOX2* in JA biosynthesis.

AtLOX2 is involved in wound-induced JA biosynthesis (Bell et al., 1995). It also has been shown that *AtLOX2* is induced by insect herbivory, and that *atlox2* antisense plants are impaired in tritrophic interactions, i.e., in induced attraction of the parasitoid *Cotesia rubecula* (van Poecke et al., 2001; van Poecke and Dicke, 2002).

In the Brassicaceae, another component of anti-herbivore defense is the glucosinolate-myrosinase system (β-thioglucoside glucohydrolase, TGG) (Rask et al., 2000; Ratzka et al., 2002; Wittstock et al., 2004). We assume that the enzyme thioglucoside glucohydrolase: myrosinase, encoded by *BoMYR,* is catalyzing the formation of glucosinolate breakdown products in *Brassica oleracea*. Myrosinase activity might even be important for plant defense against specialist herbivorous insects such as *Plutella xylostella* that have adapted to intact glucosinolates (Li et al., 2000). In addition, allyl-isothiocyanate, a glucosinolate breakdown product resulting from myrosinase activity, negatively affected another specialist herbivore, *Pieris rapae* (Agrawal and Kurashige, 2003). However, the performance of *P. xylostella* was unaffected when feeding on the *A. thaliana* myrosinase double mutant *tgg1tgg2*, while *P. rapae* even performed better on wildtype plants than on the double mutant, possibly due to reduced concentrations of feeding stimulants (Barth and Jander, 2006).

These results led us to investigate the effects of the silencing of *AtLOX2* and *AtTGG1/2* genes *via* heterologous gene sequences of *BoLOX* and *BoMYR* by applying VIGS to *A. thaliana.* Subsequently, we investigated the effects of silencing these genes on the performance of two insect herbivores, a specialist and a generalist. We found that VIGS applied to the two genes enhanced susceptibility of *Arabidopsis* to the specialist herbivore *P. rapae*, and that the effect of silencing *AtTGG1/TGG2* on the performance of the generalist *Mamestra brassicae* was even stronger This

indicates that maintaining the functions of both genes is important for plant resistance to herbivores.

Methods and Materials

Plants and Insects Seeds of the *Arabidopsis* ecotype Col-0 were sown and grown in a climate chamber at 60–70% RH, 23±1°C, 8:16 hL:D photoperiod under a light intensity of ca. 45 μmol m^{-2} sec^{-1}. Four-to-5-leaf seedlings were subjected to VIGS, approximately 21–25 d after sowing. Plants used for bioassays had been subjected to TRV-VIGS 21 d before.

Pieris rapae L. (small Cabbage White butterfly; Lepidoptera: Pieridae) and *Mamestra brassicae* L. (cabbage moth, Lepidoptera: Noctuidae) were routinely reared on cabbage plants (*B. oleracea* var. *gemmifera* cv. Cyrus) in a climate room at 21±1°C, 50–70% RH, 16:8 hL:D.

Construction of TRV Plasmids The pTRV1 (pYL192) and pTRV2 (pYL156) vectors described by Liu et al. (2002) were used in the present study. To generate lipoxygenase (*LOX*) and thioglucoside glucohydrolase: myrosinase (*TGG*) silencing VIGS plasmids, a 299-bp *BoLOX* cDNA fragment corresponding to nucleotides 1579–1877 from *B. oleracea* var. *gemmifera* cv. Cyrus cDNA (GenBank accession EF123056) (Zheng et al., 2007) and a 208-bp fragment corresponding to nucleotides 1–208 from the *BoMYR* cDNA fragment (GenBank accession DQ456999) were amplified by PCR with specific oligonucleotide primers. The following oligonucleotide primer combinations were used for amplification of *BoLOX* and *BoMYR* cDNA fragments: *BoLOX*-F1: 5'GCCATGGTGGCTATG GAATCTTGC3′ and *BoLOX*-R1: 5′CGAATTCCTCCA TAGTTTAGC3′; *BoMYR*-F1: 5′GCCATGGGCAGAAG GAAAGGTGAGTAGGGG′3 and *BoMYR*-R1: 5′ CGAATTCCTTTGAAACATAGATCCGCGTAG'3, respectively. These primers were designed with restriction enzyme recognition sites *Nco*I and *Eco*RI (underlined), respectively. The PCR products were purified and digested with *Nco*I with *Eco*RI. Subsequently, the resulting PCR products were inserted into *Eco*RI and *Nco*I-cut pTRV2, and generated antisense VIGS constructs pTRV2-*BoLOX* and pTRV2-*BoMYR*, respectively (Fig. 1). The cloned *BoLOX* fragment shares 90% nucleotide identity with the corresponding *A. thaliana* lipoxygenase (*AtLOX2*) mRNA. The cloned *BoMYR* fragment shares 79% and 80% nucleotide identity with the corresponding *A. thaliana* myrosinase *TGG1* (At5g26000) and *TGG2*(At5g25980) mRNA, respectively. Between the two *A. thaliana* myrosinase genes *TGG1* and *TGG2* there is 86% nucleotide identity. The obtained pYL156 recombinant constructs were confirmed by PCR using insert-specific primers (*BoLOX*-F1 and *BoLOX*-R1 or *BoMYR*-F1 and *BoMYR*-R1) and vector-specific primers 156F (5′- GGTCAAGGTACGTAGTAGAG −3′) and 156R (5′- CGAGAATGTCAATCTCGTAGG −3′), which span the multiple cloning site in TRV2 (Hileman et al., 2005). Both constructs then were purified, and the identity of the final constructs was verified by sequencing with the 156 F and 156R primers. Recombinant constructs were electroporated into competent cells of *Agrobacterium tumefaciens* strain AGL0 as previously described (Zheng et al., 2005) by a microPulser™ (Bio-Rad, Hercules, CA, USA). Colonies were screened by PCR using primers 156F and 156R for the presence of pTRV2-*BoLOX* or pTRV2-*BoMYR*. Glycerol stocks were made from single positive transformants.

Preparation of Agrobacterium Inoculation Cultures of *A. tumefaciens* strain AGL0 containing pTRV1, pTRV2, and pTRV2-*BoLOX* or pTRV2-*BoMYR* plasmid were cultured separately into 20 ml Luria-Betani (LB) medium containing 50 μg ml^{-1} kanamycin and 50 μg ml^{-1} rifampicin, and shaken at 28°C overnight. After centrifugation at 4000 rpm for 15 min, bacterial cell pellets were resuspended in infiltration buffer containing 1 mM MES (pH 5.8), 10 mM $MgCl_2$, and 100 μM acetasyringone to OD600−2, and allowed to stand at room temperature for 2 hr before infiltration. Suspensions of *A. tumefaciens* carrying pTRV1 and pTRV2-*BoLOX* or pTRV2-*BoMYR* were mixed 1:1 and infiltrated into the abaxial sides of the four upper leaves of 3-wk-old *A. thaliana* plants using a 2-ml syringe. To rule out an effect of an innate immunity response in silenced plants to the viral infection by the VIGS method, suspensions of *A. tumefaciens* carrying pTRV1 and pTRV2 also were mixed 1:1 and infiltrated the same way as above as empty vector control (referred to as EV control below). After agroinfiltration, *A. thaliana* plants were allowed to grow for 21 d in the same climate chamber as mentioned above.

Larval Performance of P. rapae and M. brassicae In total 12 plants from each treatment of *LOX*-silenced, *TGG*-silenced, and EV *A. thaliana* plants were offered to *P. rapae* butterflies to oviposit 5 eggs per plant. If butterflies laid more than 5 eggs on the plant, the extra eggs were removed. Another 12 intact plants in each treatment were also maintained in a climate chamber at 60–70% RH, 23±1°C, 16:8 hL:D photoperiod under a light intensity of ca. 45 μmol m^{-2} sec^{-1}. The eggs all hatched 4 d after oviposition on both plant types. The fresh body mass of individual larvae from 12 plants in each treatment was determined 7 d after oviposition (3 d of caterpillar feeding) using a CP2P Sartorius balance (Göttingen, Germany) with an accuracy of 0.001 mg.

In a second experiment, the time course of *TGG*-transcript accumulation in response to continuous larval

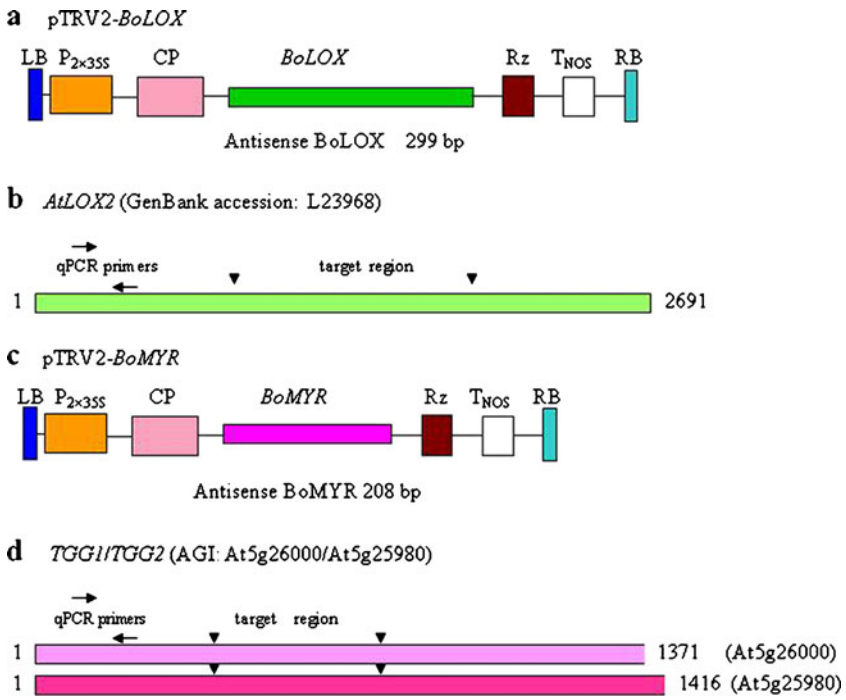


Fig. 1 Specific cDNA fragments from cabbage (*Brassica oleracea*) and TRV-based VIGS constructs used in this study. **a.** A 299-bp fragment (1579–1877 nt) from cabbage lipoxygenase (*BoLOX*) (GenBank accession EF123056) in antisense orientation cloned into TRV2 to generate pTRV2-*BoLOX*. $P_{2\times35S}$, duplicated CaMV 35S promoter; T_{NOS}; nopaline synthase terminator; CP, coat protein; LB and RB, left and right borders of T-DNA; Rz, self-cleaving ribozyme. **b.** Schematic representation of *Arabidopsis thaliana* lipoxygenase *AtLOX2* cDNA with the position of the primers (→ and ←) and size (not to scale) of amplification product used for qPCR. The fragment between two inverted triangles (▼) indicates a target region of 299 bp *AtLOX2* cDNA fragment corresponding to nucleotides 1594–1892 from *A. thaliana* cDNA by VIGS. **c.** A 208-bp fragment (1–208 nt) from cabbage myrosinase (*BoMYR*) (GenBank accession DQ456999) in antisense orientation cloned into TRV2 to generate pTRV2-*BoMYR*. **d.** Schematic representation of *Arabidopsis thaliana* myrosinase *TGG1/TGG2* cDNA with the position of the primers (→ and ←) and size (not to scale) of amplification product used for qPCR. The fragment between two inverted triangles (▼) indicates a target region of 208-bp *TGG1/2* cDNA fragment corresponding to nucleotides 368–575 (*TGG1*) or 403–610 (*TGG2*) from *A. thaliana* cDNA by VIGS

feeding was assessed in *TGG*-silenced and EV *A. thaliana* plants. Plants were offered to *P. rapae* butterflies to oviposit 5 eggs per plant. In addition, another 12 intact plants in each treatment were also maintained in the same climate chamber. This experiment was executed in another climate room at 60–70% RH, 21±1°C, 16:8 hL:D photoperiod under a light intensity of ca. 45 µmol m^{-2} sec^{-1}. Due to the lower temperature, the plants grew a little slower and eggs hatched 1 d later compared to the previous experiment, i.e., at 5 d after oviposition on both *TGG*-silenced and EV plants. Fresh body mass of individual larvae was determined at 12 h, 1, 2, and 3 d after egg hatch using a CP2P Sartorius balance; thereafter, the corresponding leaf samples were collected for quantification of *TGG* transcripts with qPCR.

For *M. brassicae,* 5 neonate larvae that hatched from eggs that had been laid on white paper were introduced on a fully developed leaf in both EV and silenced plants. Fresh body mass of individual larvae was determined after the larvae had fed for 6 d. Because neonate *M. brassicae* caterpillars have an insignificant starting weight, final weights accurately represent weight gain. There was no test of *M. brassicae* feeding on *LOX*-silenced plants, and only *TGG*-silenced plants were used.

For all experiments leaf material was collected for real-time qPCR analysis of transcript levels (see below for details).

Real-Time qPCR For real-time qPCR experiments, primer pairs were designed outside the silencing target region of the *Arabidopsis AtLOX2* or *TGG1/TGG2* cDNA, which is corresponding to its orthologous *BoLOX* or *BoMYR* fragment (Fig. 1). This ensured that only the endogenous *Arabidopsis LOX* or *TGG* mRNA, not *BoLOX* or *BoMYR* was reverse-transcribed. Because the *BoMYR* fragment is expected to target two *Arabidopsis* genes, i.e., *TGG1* and *TGG2*, based on nucleotide identity between *TGG1* and *TGG2*, we designed identical primers from these two genes to generate a 99 bp amplification product for qPCR analysis. Leaf samples for real-time qPCR reactions were harvested from individual *LOX*-silenced, *TGG*-silenced and EV plants after finishing caterpillar-development experi-

ments. Four leaves from 4 individual plants were pooled as one biological replicate and immediately frozen in liquid nitrogen for later RNA isolation. There were three biological replicates in each treatment. Total RNA extraction, purification and cDNA synthesis, primer design, qPCR reactions, and gene expression quantification were the same as described previously (Zheng et al., 2007). For the internal control, *AtGAPDH* cDNA (GenBank accession M64116) (Shih et al., 1991) was used as reference. The target gene expression relative to *AtGAPDH* expression was quantified by comparing the threshold cycle for each PCR to their respective dilution series and dividing the resulting quantities. In order to investigate whether there is any off-target of heterologous gene sequences from *B. oleracea* by *LOX* or *TGG* silencing in Arabidopsis, other endogenous *Arabidopsis* genes such as phenylalanine ammonia lyase (*AtPAL1*) (GenBank accession AY303128) (Cochrane et al., 2004) and phytoene desaturase *AtPDS* (GenBank accession L16236) (Scolnik and Bartley, 1993) also were selected for qPCR analysis beyond BLAST searching. Gene-specific primers used for quantification of gene expression are listed in Table 1. The quantification of gene expression was performed with a Rotor-gene™ 6000 (Corbett, Australia).

Data Analysis The mean values of body mass of larvae subjected to various plant treatments were analyzed by a nested ANOVA, with the plant individual as the nested term in the analysis. Gene expression values were log-transformed and statistically analyzed by a one-way ANOVA.

Results

Silencing of Endogenous AtLOX2 and AtTGG1/2 Genes in A. thaliana Plants by B. oleracea BoLOX and BoMYR Gene Sequences We used the TRV system to generate antisense VIGS constructs pTRV2-*BoLOX* and pTRV2-*BoMYR* to silence corresponding *AtLOX2* and *AtTGG1/TGG2* genes in *A. thaliana* plants (Fig. 1). There were five stretches with more than 18 nucleotides sequence identity between the *A. thaliana* and *B. oleracea LOX* cDNAs (Fig. 2a). However, only one stretch with more than 18 nucleotides in sequence identity between the *A. thaliana TGG1/TGG2* and *B. oleracea MYR* cDNAs was found (Fig. 2b). We inoculated *A. thaliana* plants with *Agrobacterium*-harboring TRV constructs that contained an empty vector (EV) or *LOX* vector (pTRV1 and pTRV2-*BoLOX*), or *MYR* vector (pTRV1 and pTRV2-*BoMYR*) to generate silenced *lox* and *tgg* plants. A positive control was included by inoculating plants with *Agrobacterium*-harboring *PDS* vector (TRV1 and TRV2-*BoPDS1*). With this positive control, we knew that photobleaching symptoms were visible in *A. thaliana* plants 10 d after agroinfiltration (Zheng et al., 2010). There were no visually distinguishable phenotypic differences among EV control, *lox*, and *tgg* plants.

We found significant differences in *AtLOX2* relative transcript abundance between different silenced plants ($F_{2,8}$= 8.59, P=0.017). *LOX*-silenced plants had significantly reduced relative *AtLOX2* transcript levels compared to EV and *TGG*-silenced plants, respectively (Fig. 3a; P=0.044 and P=0.006). There was no difference in *AtLOX2* relative transcript levels between EV and *TGG*-silenced plants (Fig. 3a). *TGG*-silenced plants had reduced *TGG1/2* relative transcript levels compared to EV plants (Fig. 3b; P=0.011). As expected, there was no difference in *AtTGG1/2* relative transcript levels between EV and *LOX*-silenced plants (Fig. 3b; P= 0.074).

In order to rule out any off-target effects of the heterologous gene sequences from *B. oleracea* by *LOX*- or *TGG*-silencing in *A. thaliana*, BLAST searching was applied, and this showed that only *A. thaliana* lipoxygenase (*AtLOX2*) mRNA or *A. thaliana* thioglucoside glucohydro-

Table 1 Lists of primers used in qPCR

Gene	Type of primer	Sequence (from 5′ to 3′)	Nucleotide position in the coding sequence
AtLOX2	Forward	ACTTGCTCGTCCGGTAATTGG	771–791 from *AtLOX2*(L23968)
AtLOX2	Reverse	GTACGGCCTTGCCTGTGAATG	929–949 from *AtLOX2*(L23968)
AtTGG1/2	Forward	CGGTGTTGCATCTTCTGCTTAC	147–168 from *TGG1* (At5g26000) 183–204 from *TGG2* (At5g25980)
AtTGG1/2	Reverse	GCTCCACCTTTCTCTGGG	228–245 from *TGG1* (At5g26000) 264–281 from *TGG2*(At5g25980)
AtGAPDH	Forward	AATGAAGGACTGGAGAGGTGGAAG	588–611 from *AtGAPDH* (M64116)
AtGAPDH	Reverse	ACGGTTGGGACACGGAAAGAC	705–725 from *AtGAPDH* (M64116)
AtPAL1	Forward	AATGGCTTCTTATTGTTCAGAGC	1410–1432 from *AtPAL1*(AY303128)
AtPAL1	Reverse	GAAGTTTTGCGAGACGAGATTAG	1510–1532 from *AtPAL1*(AY303128)
AtPDS	Forward	CAATGACGATGGCACGGTTAAGAG	1005–1028 *AtPDS* (L16237)
AtPDS	Reverse	CTGGAGCGGCAAACACATAAGC	1066–1087 *AtPDS* (L16237)



Fig. 2 Sequence alignment of specific nucleotide fragments from *Brassica oleracea*, selected to trigger gene silencing, with their respective orthologs in *Arabidopsis thaliana.* **a.** *A. thaliana* lipoxygenase *AtLOX2* (L23968) aligned with *B. oleracea BoLOX* (EF123056); **b.** There is 79% nucleotide identity between *A. thaliana* myrosinase*TGG1* (At5g26000) and *B. oleracea BoMYR* (DQ456999); 80% nucleotide identity between *A. thaliana* myrosinase *TGG2* (At5g25980) and *B. oleracea BoMYR;* 86% nucleotide identity between *A. thaliana* myrosinase *TGG1* and *TGG2*. Asterisks (*) indicate identical nucleotides. Asterisks (*) in red designate the largest identity stretches of more than 18 nucleotides between the *A. thaliana* and *B. oleracea* cDNAs

lase (*AtTGG1/TGG2*) mRNAwas targeted (data not shown). In addition, other endogenous *A. thaliana* genes such as phenylalanine ammonia lyase (*AtPAL1*) and phytoene desaturase *AtPDS* also were subjected to transcript analysis. As expected, there were no differences in *AtPAL1* and *AtPDS* relative transcript levels among EV, *LOX*-silenced and *TGG*-silenced plants (Figs. 3c, d). This further confirms that silencing *A. thaliana* plants by using the corresponding gene sequences from *B. oleracea* is specific.

Overall, these results demonstrate that different heterologous gene sequences from *B. oleracea* can be used to silence their respective orthologs in *A. thaliana* plants for plant-insect interaction studies.

Silencing AtLOX2 *or* AtTGG1/2 *Genes in* A. thaliana *Plants Results in Enhanced Feeding by Both Specialist* P. rapae *and Generalist* M. brassicae *AtLOX2* relative transcript levels differed significantly between the different types of silenced plants after 3 d of *P. rapae* herbivory (Fig. 4a; $F_{2,8}$=9.48, P=0.014). *AtLOX2* relative transcript levels were dramatically increased in EV and *TGG*-silenced plants after *P. rapae* larval feeding (Fig. 4a) as compared to the transcript levels in undamaged plants (Fig. 3a). However, after *P. rapae* damage, *LOX*-silenced plants had significantly reduced *AtLOX2* relative transcript levels compared to EV and *TGG*-silenced plants, respectively (Fig. 4a; P=0.007 and P=0.014). There was no difference in *AtLOX2* transcript levels between EV and *TGG*-silenced *P. rapae*-infested plants. As expected, *TGG1/2* transcript levels were increased in EV and *LOX*-silenced plants after *P. rapae* larval feeding. Surprisingly, there was no difference in *TGG1/2* transcript levels in *TGG*-silenced plants vs. EV and *LOX*-silenced plants after *P. rapae* larval feeding for 3 d (Fig. 4b). In order to test the time course of the effect on

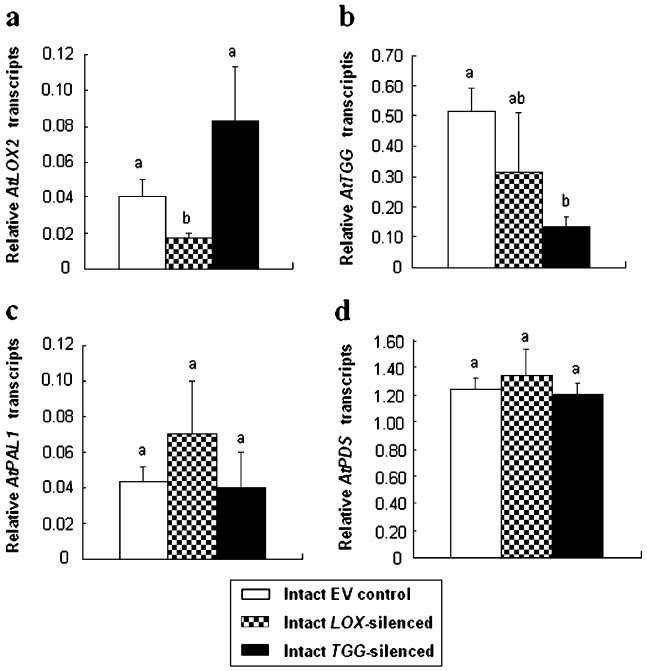


Fig. 3 Accumulation of endogenous *AtLOX2*, *TGG1/2*, *AtPAL1* and *AtPDS* transcripts relative to the levels of the *AtGAPDH* gene was analyzed by qPCR in intact *LOX*-silenced and *MYR*-silenced plants. EV denotes empty vector control plant. There were 3 biological replicates in each treatment. Different letters above bars indicate a significant difference at $P<0.05$; bars represent means and SE. One-way ANOVA was used to analyze gene expression. **a.** Silencing of endogenous lipoxygenase *AtLOX2* in *Arabidopsis thaliana* using the *BoLOX* sequence from *Brassica oleracea.* **b.** Silencing of endogenous myrosinase *TGG1/2* in *A. thaliana* using *BoMYR* sequence from *B. oleracea.* **c−d.** No off-target effects of endogenous phenylalanine ammonia lyase *AtPAL1* or phytoene desaturase *AtPDS* in *A. thaliana* by *LOX*-silenced or *TGG*-silenced plants using *BoLOX* or *BoMYR* sequences from *B. oleracea*

TGG transcripts induced by continuous larval feeding, we carried out a second experiment that employed different time periods of larval *P. rapae* feeding. There were significant differences in *TGG1/2* transcript levels between *TGG*-silenced and EV plants after larval feeding for less than 12 hr and 1 d, respectively (Fig. 5a; $P<0.05$). The reduction in *TGG1/2* transcript levels was obvious in *TGG*-silenced plants after *P. rapae* larval feeding for shorter periods compared to EV control plants. However, the differences in accumulation of *TGG1/2* transcript levels between *TGG*-silenced and EV plants disappeared after larval feeding up to 2 and 3 d, respectively (Fig. 5a). The accumulation of *TGG1/2* transcript levels in *TGG*-silenced plants after larval feeding for 3 d was consistent in these two experiments.

The body mass of larvae that fed on silenced plants was significantly enhanced compared to EV control plants. *Pieris rapae* larvae grew significantly faster on *LOX*-silenced or *TGG*-silenced plants compared to EV plants (Fig. 4c; $F_{2,35}=4.353$, $P=0.021$). Furthermore, larval performance of *P. rapae* was equal on *LOX*-silenced and *TGG*-silenced plants (Fig. 4c). In the second experiment that tested the effects of larval feeding on *TGG*-silenced plants at more time points, no significant differences in larval body mass were found after 12 h, 1 and 2 d (Fig. 5b). However, *TGG*-silencing significantly enhanced larval growth as determined after 3 d of feeding (Fig. 5b; $P<0.05$). Furthermore, after 6 d of feeding on *TGG*-silenced plants, the generalist *M. brassicae* gained 1.5 times more body weight than on EV control plants (Fig. 5c; $P<0.05$).

Discussion

Silencing Arabidopsis thaliana *Plants with Gene Sequences from* Brassica oleracea We recently reported on silencing genes in *A. thaliana, B. nigra,* and *N. benthamiana* plants by the application of TRV-mediated VIGS with a heterologous gene sequence, i.e., *BoPDS* from *B. oleracea* (Zheng et al., 2010). In the present study, we show that *BoLOX* and *BoMYR* sequences from *B. oleracea* can be used to silence their respective orthologs *AtLOX2* and *AtTGG1/2* in *A. thaliana* plants.

Silencing *AtLOX2* and *AtTGG1/2* in *A. thaliana* through sequences from the *BoLOX* and *BoMYR* genes, results in enhanced feeding by *P. rapae* larvae. In recent years, VIGS has been used successfully to unravel the functions of several plant genes belonging to the defense response pathways in the model species *N. benthamiana* and other solanaceous species like tomato and tobacco (Kandoth et al., 2007; Hartl et al., 2008; Wu et al., 2008). Despite the promise held by this strategy, there are still limitations to the way in which VIGS can be used for high-throughput functional genomics beyond these model species, mostly because of the difficulties of introducing the VIGS vector into other plant species, including the model plant *A. thaliana* (Burch-Smith et al., 2006; Wang et al., 2006; Pflieger et al., 2008). Although Senthil-Kumar et al. (2007) showed that TRV-mediated VIGS can be applied in a wide range of solanaceous plant species, and that heterologous gene sequences from distantly related plant species including *A. thaliana* can be used to silence their respective orthologs in the VIGS-efficient model plant *N. benthamiana*, the use of heterologous gene silencing in determining gene function in the context of plant-insect interactions has not been reported before.

The Function of AtLOX2 and AtTGG1/2 Silenced by BoLOX and BoMYR in Conferring Resistance to Herbivores The specific *LOX* gene that is involved in the JA biosynthesis pathway is induced in response to wounding or herbivory, and is involved in JA biosynthesis in plants such as *A.*

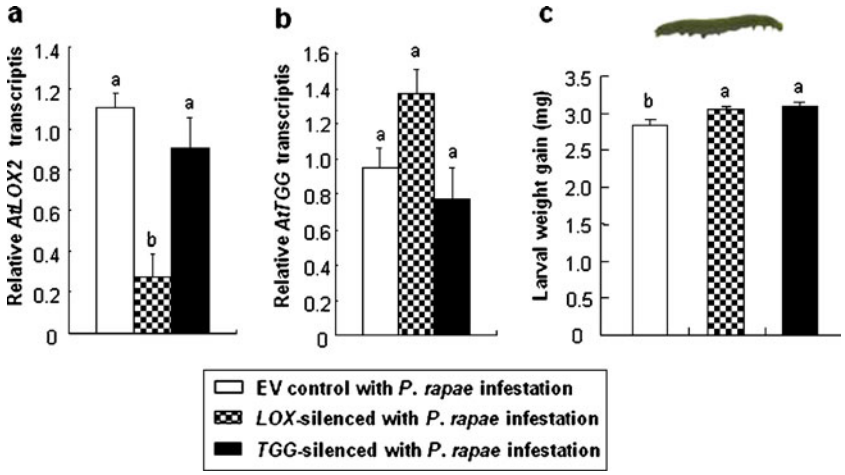


Fig. 4 Accumulation of induced *AtLOX2* and *TGG1/2* transcripts relative to the levels of the *AtGAPDH* gene as assessed by qPCR in *LOX*-silenced and *TGG*-silenced plants after 3 d of feeding by *Pieris rapae* larvae. Silencing *AtLOX2* or *TGG1/2* enhances *P. rapae* larvae performance in *Arabidopsis thaliana* plants. There were 3 biological replicates in each treatment. Different letters above bars indicate significant difference at $P<0.05$; bars represent means and SE. One-way ANOVA and a nested ANOVA were used to analyze gene expression and body mass, respectively. **a.** Silencing lipoxygenase in *A. thaliana* using a partial *BoLOX* sequence from *B. oleracea* significantly reduced *AtLOX2* transcripts after 3 d of feeding by *P. rapae* larvae. **b.** Silencing myrosinase *TGG1/2* in *A. thaliana* using a *BoMYR* sequence from *Brassica oleracea* did not significantly reduce *TGG1/2* transcript levels after 3 d of feeding by *P. rapae* larvae. **c.** Growth of *P. rapae* larvae ($F_{2,35}=4.353$, $P=0.021$) on different silenced plants in no-choice feeding experiment 3 d after caterpillar emergence from the egg (each genotype with 12 plants and 5 eggs per plant; larvae hatched 4 d after oviposition). EV denotes empty vector control plant. The leaf samples were harvested 7 d after *P. rapae* oviposition

thaliana, tobacco, rice, and tomato (Bell et al., 1995; Heitz et al., 1997; Halitschke and Baldwin, 2003; Zhou et al., 2009; Allmann et al., 2010). Disrupting this specific *LOX* mRNA accumulation, e.g., through antisense as-*lox* in tobacco and rice results in a plant phenotype that is impaired in defense against herbivorous insects such as caterpillars (Halitschke and Baldwin, 2003; Kessler et al., 2004; Zhou et al., 2009; Allmann et al., 2010). *AtLOX2* induction occurs upon insect herbivory and *AtLOX2* antisense plants are impaired in anti-insect defense (van Poecke et al., 2001; van Poecke and Dicke, 2002). We previously hypothesized that *BoLOX* is characteristic for a 13-lipoxygenase (13-LOX) involved in JA biosynthesis (Zheng et al., 2007). In this study, we demonstrated that a *BoLOX* sequence can silence *AtLOX2* in *Arabidopsis.* This indicates that there is sufficient identity between *BoLOX* and *AtLOX2* to mediate gene silencing and *BoLOX*, like *AtLOX2,* may be involved in the jasmonate signaling pathway and responsible for wound-induced JA biosynthesis (see also Zheng et al., 2007) which should be further proven by silencing *BoLOX* in *B. oleracea.* At present, however, silencing in *B. oleracea* is not yet sufficiently effective (Zheng, unpublished data).

In the Brassicaceae family, the glucosinolate-myrosinase system serves as the major chemical defense mechanism against insects (Rask et al., 2000; Ratzka et al., 2002; Wittstock et al., 2004). The enzyme myrosinase degrades glucosinolates to produce toxins such as isothiocyanates that deter herbivory. A role of isothiocyanates in *A. lyrata* and *B. oleracea* resistance against the specialist herbivore *P. rapae* has been suggested by incorporating micro-encapsulated isothiocyanate in an artificial diet (Agrawal and Kurashige, 2003). However, *P. rapae* larvae performed better on wild-type *A. thaliana* than on double mutant *tgg1tgg2* plants, ascribed to very low myrosinase activity and reduced levels of feeding stimulants (Barth and Jander, 2006). Our results partially agree with the conclusions of Barth and Jander (2006), who report that elimination of TGG activity results in an increase in mass in the Solanaceae-specialist *Manduca sexta,* and the generalist *Trichoplusia ni,* a decrease in mass gain in *P. rapae*, while they recorded no effects on mass gain in the specialist *Plutella xylostella.* Our results show that endogenous *TGG1/2* transcript levels in intact *TGG*-silenced plants were reduced by about 80% compared to EV plants (Fig. 3b; Fig. 5a). As a consequence, *P. rapae* larval growth was significantly enhanced after feeding for 3 d on silenced plants compared to feeding on EV plants (Fig. 4c; Fig. 5b) which supports the conclusions drawn by Agrawal and Kurashige (2003), i.e., that glucosinolate breakdown products such as isothiocyanates that are a result of myrosinase activity confer resistance to the crucifer specialist *P. rapae*. There were significant differences in the accumulation of *TGG1/2* transcript levels between *TGG*-silenced and EV plants after larval feeding for periods of only <12 hr and 1 d, respectively (Fig. 5a). However, no

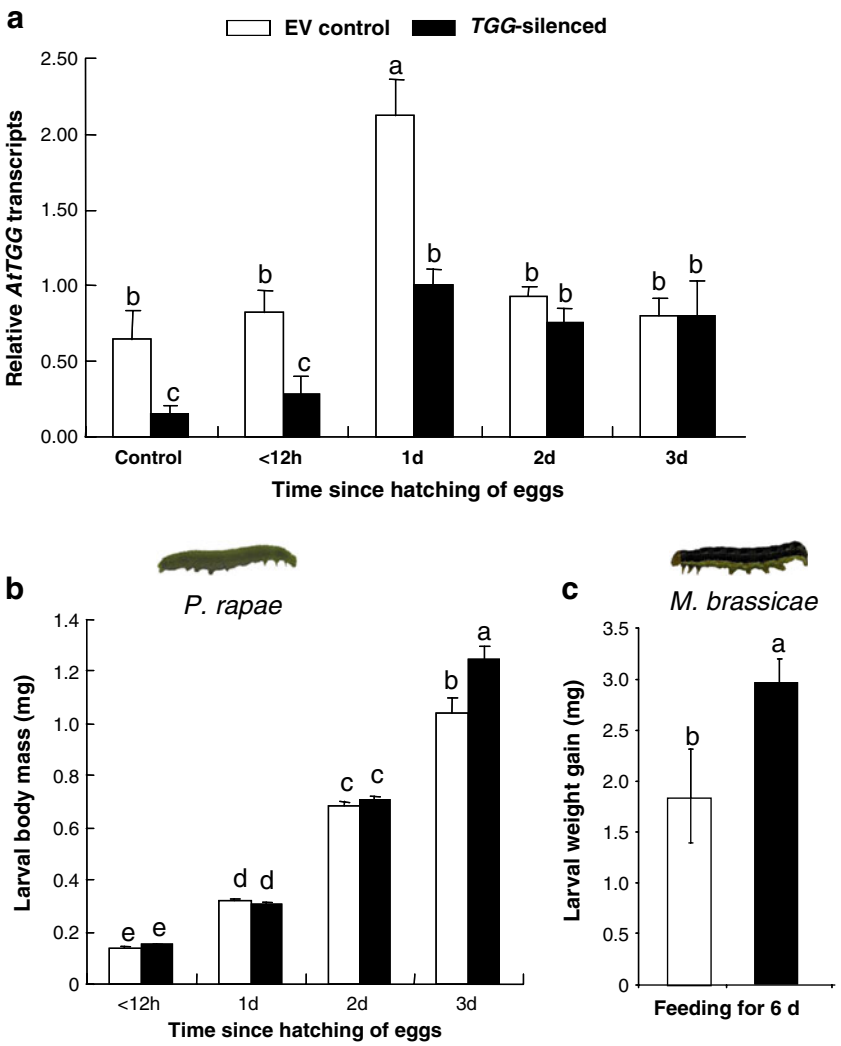


Fig. 5 Time course of accumulation of induced *TGG1/2* transcripts relative to the levels of the *AtGAPDH* gene and performance of *Pieris rapae* larvae on myrosinase *TGG1/2* silenced and empty vector control plants. There were 3 biological replicates in each treatment. Different letters above bars indicate significant difference at $P<0.05$; bars represent means and SE. One-way ANOVA and a nested ANOVA were used to analyze gene expression and body mass, respectively. **a**. Silencing myrosinase *TGG1/2* in *Arabidopsis thaliana* using *BoMYR* sequence from *Brassica oleracea* significantly reduces *TGG1/2* transcript levels in response to feeding by *P. rapae* larvae during less than 12 hr and 1 d, respectively. **b.** Silencing *TGG1/2* enhances *P. rapae* larval performance in *A. thaliana* plants in response to feeding by *P. rapae* larvae during 3 d, but not at earlier time points. Growth of *P. rapae* larvae on silenced or EV plants in a no-choice feeding experiment lasting 3 d after egg hatch. **c.** Silencing *TGG1/2* enhances growth of larvae of the generalist *Mamestra brassicae* after feeding for 6 d on *A. thaliana* plants. EV denotes empty vector control plant. The leaf samples were harvested at different time points as indicated as above

significant differences in *TGG1/2* transcript levels were detected upon *P. rapae* larval feeding for 2 and 3 d, respectively (Fig. 5a). This indicates that silencing *TGG1/2* genes has a delayed effect on larval development (Fig. 5b), which may be the result from a delay from transcription to translation that allows the effect to be noticeable. It would be interesting to measure additional parameters e.g., time to pupation, adult size, sex ratio, etc. However, due to the small size of the *A. thaliana* plants, five larvae could not complete development on a single plant. Furthermore, it will be interesting to quantify myrosinase protein levels, myrosinase activity, and glucosinolate breakdown products in future studies to assess the effects of changes in transcription level on the glucosinolate phenotype directly. The *P. rapae* caterpillars hatched from eggs that had been laid on the experimental plants. It is known that egg deposition may induce plant genes (Fatouros et al., 2008), and that extracts of crushed eggs may suppress the induction of plant defense genes (Bruessow et al., 2010). The *M. brassicae* caterpillars hatched from eggs that had been laid on paper. Our results show that in both cases caterpillar feeding induced a defense. Collectively, based on the comprehensive bioassays and gene expression studies, our results demonstrate an important role of *AtLOX2* and *AtTGG1/2* genes in *A. thaliana* plant resistance to the specialist and generalist herbivores *P. rapae* and *M. brassicae,* respectively.

Nucleotide Sequence Length Constraints for Heterologous Silencing Interestingly, we found that the overall homology between the heterologous gene sequence and the target gene sequence does not always correlate with gene silencing efficiency. A 299-bp *BoLOX* cDNA fragment used as trigger in the *LOX* vector has 90% of its nucleotides identical with the corresponding *AtLOX2* cDNA target fragment (Fig. 2a). However, intact *LOX*-silenced plants showed a reduction of approximately 50% in the abundance of *AtLOX2* transcripts compared to EV plants (Fig. 3a).

Moreover, intact *TGG*-silenced plants showed a reduction of approximately 80% in the abundance of *TGG1/2* transcripts compared to EV plants, although a 208-bp *BoMYR* cDNA fragment as trigger in *MYR* vector has only 79–80% identical nucle otides with the corresponding *AtTGG1/2* cDNA target fragment (Fig. 2B; Fig. 3B). Surprisingly, our data show that only 18 nucleotides (TACGCGGATC TATGTTTC) with 100% identity between the trigger and the target sequence are sufficient to achieve effective gene silencing (Fig. 2B). After BLAST searching of these 18 nucleotides and qPCR data on two other genes, *AtPAL1* and *AtPDS,* we did not find any off-target but only an effect on the target gene that codes for thioglucoside glucohydrolase (*AtTGG1/TGG2*) (data not shown). It has been reported that a minimum length of 21–23 nucleotides with 100% identity between the trigger and the target sequence is required to cause gene silencing (Zamore et al., 2000; Thomas et al., 2001; Voinnet, 2001). A 21-nucleotide stretch of 100% identity between the heterologous and endogenous gene sequences is not absolutely required for *PDS* gene silencing in *N. benthamiana* (Senthil-Kumar et al., 2007). Our results show that in *A. thaliana* another functional gene silencing can be achieved without the 21-nucleotide minimum length. Furthermore, the silenced *tgg* plants enhance larval performance of the specialist *P. rapae* and, more strongly so, of the generalist *M. brassicae* after 3 and 6 d, respectively, of feeding since egg hatch (Fig. 4C; Fig. 5B, C). It is important to target specific genes while avoiding off-target effects when using such short homologous gene sequences.

VIGS is a useful strategy for studying gene functions in plant-insect interactions, and EV plants were included to rule out an effect of innate immunity response in silenced plants to the viral infection. The next step is to develop VIGS successfully in *B. oleracea.* Especially, because there are no collections of genome-wide mutant populations generated by T-DNA insertion and transposon tagging in *Brassica* species. Generation of VIGS in *B. oleracea* has turned out to be more difficult than in *A. thaliana* (Zheng et al., 2010). Our present results address the use of heterologous gene sequences from the cultivated crop species *B. oleracea* to silence their respective orthologs in the model plant *A. thaliana*. This method is a first step in studies on the possible roles of *B. oleracea* genes by triggering gene silencing of corresponding heterologous genes in *Arabidopsis* plants for plant-insect interactions. Developing new protocols to improve silencing efficiency in *Brassica* species themselves will be the next step in our research to investigate the function of the *Brassica* genes. Furthermore, VIGS holds promise to silence particular genes of interest at different levels of silencing stringency. Linking different gene expression levels with quantified phenotypic plant traits will be vital to unravel mechanisms underlying plant-insect interactions (Zheng and Dicke, 2008).

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Are the Phytoestrogens Genistein and Daidzein Anti-Herbivore Defenses? A Test Using the Gypsy Moth (*Lymantria dispar*)

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Abstract Phytoestrogens are compounds that have moderate estrogenic or anti-estrogenic activity toward mammals. Although genistein and daidzein, the main phytoestrogens of soybean, have been the subject of thousands of studies that address their benefit to human health, relatively little is known about their benefits to plants that produce them. It has been suggested that genistein and daidzein protect plants against arthropod herbivores, but direct tests of this hypothesis are rare. In this study, we evaluated the effect of genistein and daidzein on the survivorship, growth, and fecundity of the gypsy moth, a generalist insect herbivore that does not encounter phytoestrogens in its normal diet. We compared survivorship, egg-to-pupa growth rate, and 4th instar performance of gypsy moth caterpillars on artificial diets containing no phytoestrogen, genistein, daidzein, or a combination of genistein and daidzein. Our results indicate that genistein and daidzein do not decrease survivorship, growth, or fecundity of this insect herbivore. Therefore, it seems unlikely that the primary function of these compounds in aboveground plant tissues is anti-herbivore defense.

Key Words Genistein · Daidzein · Phytoestrogen · Gypsy moth · Larval performance · Anti-herbivore defense

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Introduction

Phytoestrogens are plant compounds that, because of their structurally similarity to estradiol, have moderate estrogenic or anti-estrogenic activity toward mammals. Phytoestrogens occur in over 300 species of plants, most notably legumes (Tham et al., 1998). Genistein and daidzein (Fig. 1), the primary phytoestrogen isoflavones of soybean, have been the subject of thousands of studies that address their beneficial effects on human health, including hormone-dependent breast and prostate cancers, osteoporosis, cardiovascular disease, immunity and inflammation, and reproduction and fertility in humans and other mammals (Cornwell et al., 2004; Xiao, 2008; Andres et al., 2009; Baber, 2010; Bhupathy et al., 2010).

Phytoestrogens have been proposed to confer three types of benefit to plants: facilitation of associations with below-ground mutualists including nitrogen-fixing bacteria and mycorrhizal fungi; protection against pathogens; and protection against herbivores. The first two are well-documented. Numerous studies have demonstrated the role of phytoestrogens in attracting and promoting colonization by mutualistic bacteria (Schultze and Kondorosi, 1998; Makoi and Ndakidemi, 2007; Bhattacharya et al., 2010) and fungi (Requena et al., 2007; Badri et al., 2009). It is also well established that the phytoalexin glyceollin, for which daidzein is a precursor, is induced by and protects plants against fungal pathogens (Lozovaya et al., 2007; Jasinski et al., 2009; Lygin et al., 2010). However, the efficacy of phytoestrogens as anti-herbivore compounds is far less clear.

Only a few studies have addressed the potential anti-herbivore benefits of phytoestrogen isoflavones, and most of these provide only indirect evidence. For instance, soybean extracts containing genistein and/or daidzein possess antifeedant or antibiotic activity toward mites

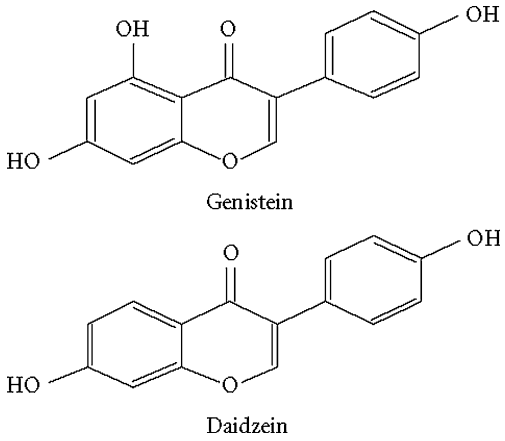


Fig. 1 Genistein and daidzein

(Wang et al., 1999), stink bugs (Piubelli et al., 2003), and lepidopterans (Hoffmann-Campo et al. 2001; Piubelli et al., 2005). However, as the authors note, these extracts contained many compounds, so it is not possible to unequivocally attribute deterrence or antibiosis to genistein and daidzein.

More convincing, but far less common, are controlled studies in which genistein and/or daidzein are applied to a neutral medium. Unfortunately, even in such controlled studies, evaluation of the anti-herbivore effects of genistein and daidzein is complicated by uncertainty about the ecological relevance of doses used (Sutherland et al., 1980; Sharma and Norris, 1991).

In a recent review of insect-soybean interactions, Parrott et al. (2008) called attention to "a notable lack of supportive research" into the role of phytoestrogens as deterrents to herbivory. We report here the results of a controlled study in which a generalist herbivore, the gypsy moth, was exposed to naturally-occurring levels of the two most abundant soybean phytoestrogens, genistein and daidzein.

Methods and Materials

The gypsy moth (*Lymantria dispar*) is a generalist herbivore that feeds on leaves of deciduous trees. Because gypsy moths do not feed on phytoestrogen-containing plants, we considered it to be particularly likely to be sensitive to genistein and/or daidzein.

Gypsy moth caterpillars were reared from hatching through the end of the final instar on gypsy moth artificial diet; both gypsy moth eggs and diet were generously provided by V. Mastro and C. Lokerson of the U.S.D.A. Plant Protection and Quarantine lab in Otis, MA, USA. Genistein and daidzein, the two major phytoestrogens in soybean (Liggins et al., 2000; Zhu et al., 2005), were purchased from LC Laboratories (Woburn, MA, USA). The rearing diet contained no phytoestrogen (control), 0.84 mg/g dry weight genistein, 0.84 mg/g dry weight daidzein, or 0.42 mg/g dry weight of both genistein and daidzein. These levels were chosen to reflect the highest reported concentration of genistein in any aboveground plant tissue (soybeans) (Dixon, 2004), and are substantially higher than levels found in leaves (Mazur and Adlercreutz, 1998).

Effect of Phytoestrogens on Survivorship To determine whether genistein and/or daidzein affects gypsy moth survivorship, 10 newly hatched larvae from each of 28 full-sib families were placed together into each of four 25-ml polystyrene cups in an environmental chamber at 30°C with a 16:8 hL:D cycle (N=1120 larvae). Each cup contained one of the four diets (control, genistein, daidzein, or genistein+ daidzein). Larvae were given fresh diet every 3 d. Upon molting into the 3rd instar, the 10 larvae from each family were divided equally between two cups to ensure sufficient space for the insects.

The first two larvae from each family on each diet that molted into the 4th instar were removed and used as wet/dry standards for the nutritional index study described below, and the fifth larva was used to measure nutritional indices. The third,fourth, sixth, and seventh larvae from each family on each diet that molted into the 4th instar were reared through to pupation on their respective diets (N=448 larvae; hereafter "egg-to-pupa larvae"). The remaining 3 larvae were discarded. After molting into the 4th instar, the 4 egg-to-pupa larvae were placed together in an 11-cm Petri dish and provided fresh food every 3 d or more often if necessary until pupation.

Early instar survivorship, defined as survivorship from hatching through the 3rd instar, was measured for all larvae, while late instar survivorship, defined as survivorship from the 4th instar through pupation, was measured only for the 448 egg-to-pupa larvae. Since larvae reared in the same cup are not independent replicates, early instar survivorship was first calculated for the 10 larvae from the same family on a given diet. Since survivorship was always at least 80%, differences among diets in early survivorship were determined by comparing the number of families with 100% vs. 80–90% survivorship by a *Chi-square* test. Similarly, differences among diets in late instar survivorship were determined by comparing the number of families with 100% vs. 75% survivorship by a *Chi-square* test. For both early and late instar survivorship, a Yates correction was applied when one or more expected value was less than 5.

Effect of Phytoestrogens on Growth The effect of genistein and daidzein on gypsy moth growth was determined in two ways. Egg-to-pupa growth rate (EPGR) was calculated for each of the 442 egg-to-pupa larvae that survived to pupation by drying pupae to constant weight at 60°C and

dividing the final pupal dry weight by the number of days from hatching to pupation. The initial dry weight of all larvae was assumed to be negligible. Upon pupation, the fresh weight of each pupa was also measured, and compared to the pupal dry weight to calculate pupal percent water. Since larvae reared in the same dish are not independent replicates, all measures were averaged separately for males and females sharing a dish (i.e., larvae of the same sex and from the same family on a given diet), and averages were compared among diets by ANOVA or, if data were not normal or variances were unequal, by Kruskal-Wallis tests. Sample sizes are slightly less than 28 because, on each diet, at least one dish yielded only male or female pupae.

Relative growth rate during the 4th instar and its components (consumption rate, digestive efficiency, and conversion efficiency) were measured for one larva per family per diet. As described above, the fifth larva from each family on each diet to molt to the 4th instar was used to in this experiment (N=28 larvae per diet). We chose the 4th instar since this is the last instar that does not end with pupation for either sex; males pupate after five instars and females pupate after six instars.

Upon molting into the 4th instar, each test larva was weighed and placed into a new 25-ml polystyrene cup containing a weighed amount of control, genistein, daidzein, or genistein+daidzein diet. Fresh diet was provided every other day. Upon molting into the 5th instar, each larva was weighed, frozen, dried to constant weight at 60°C, and reweighed. All uneaten food and all frass produced during the instar were also dried and weighed. Larval performance measures (Waldbauer, 1968; Karowe and Golston, 2007), based on dry weights, were calculated as follows:

$$\text{Relative consumption rate(RCR)} = \text{food ingested}/(\text{average larval weight})(\text{days})$$

$$\text{Approximate digestibility(AD)} = (\text{food ingested} - \text{frass})/\text{food ingested}$$

$$\text{Efficiency of conversion of digested food(ECD)} = \text{weight gained}/(\text{food ingested} - \text{frass})$$

$$\text{Efficiency of conversion of ingested food(ECI)} = \text{AD} \times \text{ECD}$$

$$\text{Relative growth rate (RGR)} = \text{weight gained}/(\text{average larval weight})(\text{days})$$

The effect of diet on nutritional indices and other measures of 4th instar performance (instar duration, final dry weight, and weight gained and frass produced during the 4th instar) was determined by one-way ANOVA followed by Tukey pairwise comparisons if data were normal and variances were equal, or by Kruskal-Wallis tests followed by pairwise Mann–Whitney U test if the assumptions of ANOVA were not met.

Results

Effect of Phytoestrogens on Survivorship Early instar survivorship ranged from 97.5% to 100% and did not differ significantly among the four diets (χ^2=6.89, $d.f.$=3, P=0.075; Fig. 2a). Late instar survivorship ranged from 99.1% to 100%. Since only a single late instar larva died, no statistical analysis was possible; however, it is clear that late instar survivorship did not differ among diets (Fig. 2b). It is unlikely that inclusion of the three discarded larvae per family per diet would have resulted in differences among diets. Although these were the slowest growing larvae, the great majority were growing nearly as well as their siblings. Across diets, only 0% to 3.7% of discarded larvae were in the 2nd instar, while 96.3% to 100% were in the 3rd instar (χ^2=3.4, $d.f.$=3, P>0.3).

Effect of Phytoestrogens on Egg-to-Pupa Growth Among females, measures of egg-to-pupa performance did not differ significantly among diets (Table 1). Egg-to-pupa growth rate ranged from 7.45 to 7.79 mg/day (P=0.49), the number of days from hatching to pupation ranged only from 53.9 to 54.5 (P=0.35), pupal dry weight ranged from 40.3 to 42.3 mg (P=0.45), and pupal water content ranged only from 77.2 to 77.6% (P=0.38).

Similarly, among males, most measures of egg-to-pupa performance did not differ among diets (Table 1). Egg-to-pupa growth rate ranged from 2.34 to 2.49 mg/day (P=0.41), the number of days from hatching to pupation ranged only from 48.0 to 48.7 (P=0.76), and pupal dry weight ranged from 11.3 to 12.1 mg (P=0.39). However, the water content of male pupae differed among diets (χ^2=8.16, $d.f.$=3, P=0.043). Water content was significantly higher on the genistein+daidzein diet than on the control, genistein, and daidzein diets.

Effect of Phytoestrogens on 4th Instar Performance Measures of 4th instar performance did not differ among larvae reared on control vs. phytoestrogen-containing diets (Table 2). No effect of diet was observed for any of the nutritional parameters, which generally differed by less than 5% among diets. Larger, but still nonsignificant, differences were noted for final dry weight, which differed by 17%

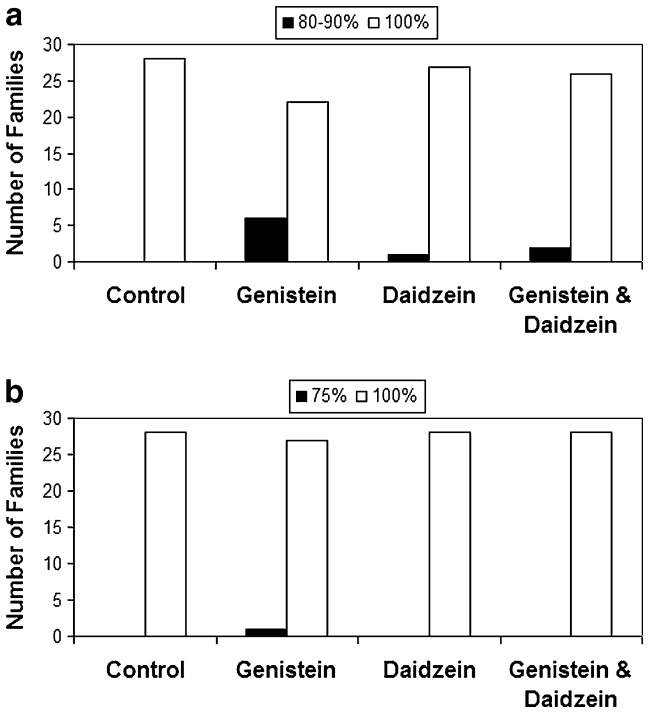


Fig. 2 (**a**, *top*) Early survivorship of gypsy moth (through the 3rd instar), expressed as the number of families on each diet that had 80–90% vs. 100% survivorship and (**b**, *bottom*) late survivorship (4th instar through pupation), expressed as the number of families on each diet that had 75% vs. 100% survivorship

among diets, total weight gained during the feeding trial, which differed by 20% among diets, and total food eaten during the feeding trial, which differed by 24% among diets.

Discussion

The results of this study indicate that the two most widely studied phytoestrogens, genistein and daidzein, have no dietary effect on a generalist herbivore, the gypsy moth. Even when these compounds were incorporated into an artificial diet at levels far above those normally found in leaves, gypsy moth survivorship was nearly 100%. Moreover, none of the measures of performance during the entire larval stage or during the 4th instar were compromised by genistein or daidzein, alone or in combination. Therefore, the results of this study do not support the hypothesis that these phytoestrogens occur in aboveground plant tissues and defend against generalist insect herbivores.

Previous studies have detected antifeedant and/or antibiotic effects of genistein and/or daidzein. For instance, when applied to agar-cellulose disks, genistein reduced consumption by the root-feeding scarab beetle, *Heteronychus arator* (Sutherland et al., 1980). Similarly, when applied to leaf disks, daidzein adversely affected growth and survivorship of the cabbage looper, *Trichoplusia ni* (Sharma and Norris, 1991). Unfortunately, since the weight of disks was not reported, it is not possible to determine whether these herbivores ingested phytoestrogens at levels known to occur in nature. We are aware of only one study in which it is possible to calculate exposure level. Wang et al. (1998) observed deterrence of redlegged earth mites, *Halotydeus destructor*, by a genistein-containing solution, but only at concentrations of 0.045% and above. Assuming the test solution had a density of approximately 1 g/ml, this concentration is more than five times the maximum genistein level reported from soybeans (Mazur and Adlercreutz, 1998; Dixon, 2004). It is not clear that adverse effects previously reported in the literature provide strong evidence of anti-herbivore effects of genistein or daidzein.

Consistent with our results, several studies have suggested that genistein does not possess anti-herbivore activity. For instance, genistein did not reduce feeding by scarab beetles *Costelytra zealandica* (Sutherland et al., 1980; Lane et al., 1987) or *Heteronychus arator* (Lane et al., 1987). In a related study (Lane et al., 1985), genistein did not reduce feeding by *C. zealandica* when incorporated into agar-cellulose disks at 0.2 mg/g, a concentration known to occur in soybean (Dixon, 2004). The authors suggested that this lack of activity might be due to the absence of an oxy function at the 2′ position of the B ring and, even more importantly, the absence of a B ring cyclic isoprenoid moiety.

It should be noted that most flavonoids and isoflavonoids exist in plant tissues primarily as glycosides rather than as aglycones such as genistein and daidzein. However, it is likely that in insects, as in humans, glycosides are readily converted into aglycones by β-glucosidases in the gut (Matsuura and Obata, 1993; Steensma et al., 1999) although that conversion has not been demonstrated for insects.

If genistein and daidzein do not provide effective defense against insect herbivores, then why are they present in aboveground plant parts? Phytoestrogens may defend plants by reducing fertility in herbivorous mammals (Jefferson et al., 2009) or seed-eating birds (Rochester and Millam, 2009), or by conferring resistance to fugal (Naoumkina et al., 2007) or viral (Andres et al., 2009) pathogens.

It is premature to rule out the possibility that phytoestrogens are in some cases effective defenses against insect herbivores. There was a slight indication in this study that genistein may decrease early instar survivorship (P=0.075; Fig. 2a), although the increase in mortality was very small. Furthermore, since it is highly polyphagous, the gypsy moth may be less susceptible than most insect herbivores to adverse effects of phytoestrogens. Although there is no reason to believe that gypsy moth encounters genistein or daidzein in its natural diet, it

Table 1 Egg-to-pupa performance (Mean±S.D.) for female and male gypsy moth larvae reared on control or phytoestrogen-containing diets

Females[a]					
Diet	*N*	Egg-to-Pupa Growth Rate (mg/d)	Development Time (days)	Pupal Dry Weight (mg)	Water Content (%)
Control	25	7.45±0.76[a]	54.2±1.6[a]	40.3±4.2[a]	77.6±0.7[a]
Genistein	27	7.77±0.94[a]	54.5±1.4[a]	42.3.±4.5[a]	77.2±0.8[a]
Daidzein	23	7.63±0.84[a]	53.9±1.4[a]	41.1±4.7[a]	77.5±1.0[a]
Genistein+Daidzein	26	7.79±0.96[a]	53.9±1.2[a]	41.9±4.8[a]	77.5±0.9[a]
$F_{3,97}$		0.82	1.11	0.88	1.05
P-value		0.49	0.35	0.45	0.38
Males					
Diet	*N*	EPGR (mg/d)	Development Time (days)	Pupal Dry Weight (mg)	Water Content (%)[a]
Control	25	2.34±0.30[a]	48.0±3.1[a]	11.3±1.8[a]	79.3±2.0[a]
Genistein	25	2.49±0.36[a]	48.7±2.7[a]	12.1±1.9[a]	79.0±1.4[a]
Daidzein	27	2.47±0.35[a]	48.2±1.9[a]	11.9±1.9[a]	79.1±1.9[a]
Genistein+Daidzein	26	2.46±0.34[a]	48.5±3.0[a]	11.6±2.3[a]	78.4±2.3[b]
Test statistic[b]		0.96	0.39	1.01	8.16
P-value		0.41	0.76	0.39	0.043

[a] Means followed by the same letter are not significantly different at $P<0.05$

[b] Significance of differences determined by a Kruskal-Wallis test followed by pairwise Mann–Whitney U tests since data could not be normalized

[c] F statistic for ANOVA or χ^2 statistic for Kruskal-Wallis test

Table 2 Nutritional indices (Mean±S.D.) for 4th instar gypsy moth larvae fed control or phytoestrogens-containing diets

Diet	*N*	RCR (g/g/d)[a]	AD (%)[a]	ECD (%)[a]	ECI (%)[a]	RGR (g/g/d)
Control	22	0.438±0.086	51.6±5.6	60.4±11.4	30.6±3.6	0.132±0.013
Genistein	25	0.416±0.039	51.6±4.1	61.5±6.7	31.5±1.5	0.131±0.011
Daidzein	23	0.419±0.046	50.8±2.7	62.1±6.0	31.8±2.8	0.133±0.014
Genistein+ Daidzein	23	0.437±0.058	50.6±5.2	60.4±4.3	30.4±2.8	0.132±0.009
Test statistic[b]		1.72	0.95	1.28	2.41	0.13
P-value		0.63	0.81	0.73	0.49	0.94

Diet	*N*	4th Instar Duration (days)	Final Dry Weight (g)[a]	Weight Gained (g)[a]	Food Eaten (g)[a]
Control	22	9.13±0.84	0.059±0.019	0.045±0.017	0.149±0.062
Genistein	25	9.02±0.76	0.057±0.017	0.042±0.014	0.135±0.046
Daidzein	23	9.13±0.84	0.054±0.015	0.041±0.013	0.131±0.041
Genistein+ Daidzein	23	9.35±0.78	0.063±0.017	0.049±0.014	0.162±0.052
Test statistic		0.69	3.57	2.45	4.58
P-value		0.56	0.31	0.48	0.21

[a] Significance of differences determined by a Kruskal-Wallis test because data could not be normalized

[b] *F* statistic for ANOVA or χ^2 statistic for Kruskal-Wallis test

RCR Relative consumption rate

AD Approximate digestibility

ECD Efficiency of conversion of digested food

ECI Efficiency of conversion of ingested food

RGR Relative growth rate

does encounter the structurally similar compounds rutin and quercetin (Liebhold et al., 1995; Perić-Mataruga et al., 1997). However, since both rutin and quercetin inhibit development of gypsy moth caterpillars (Beninger and Abou-Zaid, 1997; Peric-Mataruga et al., 2001), it is unlikely that gypsy moths possess a broad ability to detoxify isoflavones. Our results, however, indicate that genistein and daidzein do not affect this insect herbivore. Therefore, it seems unlikely that the primary function of these compounds in aboveground plant tissues is deterrence of generalist herbivores.

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Palatability and Chemical Defense of *Phragmites australis* to the Marsh Periwinkle Snail *Littoraria irrorata*

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Abstract Coastal marsh habitats are impacted by many disturbances, including habitat destruction, pollution, and the introduction of invasive species. The common reed, *Phragmites australis*, has been particularly invasive in the mesohaline regions of the Chesapeake Bay, but few studies have investigated its role in trophic interactions with North American marsh consumers. The marsh periwinkle snail *Littoraria irrorata* is a common grazer in marshes and grazes on the native grass *Spartina alterniflora*. Whether this snail grazes on *Phragmites* has not been addressed. We found *Spartina* leaves to be tougher than those of *Phragmites*, but despite this, snails consumed significantly more *Spartina* than *Phragmites*. Subsequent experiments demonstrated that *Phragmites* is chemically deterrent to snails by an unknown, moderately polar, compound. Further studies are required to more fully understand the interactions between *Phragmites*, herbivores, and *Spartina*, and how they may impact marsh ecosystems.

Key Words Estuarine · Exotic · Non-native · Plant-herbivore · Resistance to herbivory

Introduction

Tidal marsh habitats provide critical ecological services, such as buffering coastlines from wave action, filtering out sediments and pollutants, and serving as nursery habitats for commercially important species (Pennings and Bertness, 2001; reviewed in Gedan et al., 2009). Increasingly, marshes are suffering from a number of negative impacts, such as land use changes, pollutants, and eutrophication, all of which can alter species richness and abundance (Burdick and Konisky 2003; Jeffries et al., 2003; Gedan et al., 2009). Another stressor on marshes is the introduction of invasive species, which can decrease the species richness and abundance of native species through activities such as consumption (e.g., nutria, *Myocaster coypus*), burrowing (e.g., isopod, *Sphaeroma quoyantum*), and competition (e.g., common reed, *Phragmites australis*) (Ford and Grace, 1998; Talley et al., 2001; Burdick and Konisky, 2003).

Phragmites australis (Cav.) Trin. ex Steud., hereafter referred to as '*Phragmites*,' is an exotic marsh grass originally from Eurasia. It is found from freshwater to marine salinities along much of the Atlantic and Gulf coasts of the U.S. It is increasing in abundance in oligohaline and mesohaline regions of the Chesapeake Bay (Cross and Fleming, 1989; Rice et al., 2000; McCormick et al., 2010). As an example, in the Rhode River subestuary of the Bay, *Phragmites* coverage increased by about 2400% between 1971–2007 (McCormick et al., 2010). *Phragmites* was introduced to North America in the early nineteenth century, but went largely unnoticed, because there are several varieties (haplotypes) of *Phragmites* native to North America that are not invasive (Saltonstall, 2002). Invasion by *Phragmites* is correlated with decreased abundance of the most common native marsh grass *Spartina alterniflora* (hereafter referred to as '*Spartina*'), as well as native *Phragmites* haplotypes, and other vascular plant species (Chambers et al., 1999; Rice et al., 2000; Saltonstall, 2002). Thus, it has been inferred that *Phragmites* is responsible for the observed declines in native species. Replacement of *Spartina* by *Phragmites* generally increases marsh elevation and decreases the number of water-filled depressions and

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the amount of standing water (Able and Hagan, 2003). In addition, the frequency of flooding, flooding depth, and duration may decrease as well (Osgood et al., 2003).

Several studies have investigated differences in associated fauna in *Spartina*- vs. *Phragmites*-dominated marsh, with mixed results. Some studies have found fewer fish, blue crabs, and macroinfaunal organisms, and differences in types of arthropods in *Phragmites*-dominated marshes, compared to marshes dominated by *Spartina* (Hanson et al., 2002; Jivoff and Able, 2003; Posey et al., 2003; Gratton and Denno, 2006). Other studies, however, have not detected faunal differences between marsh types (Hanson et al., 2002; Able and Hagan, 2003; Osgood et al., 2003; Raichel et al., 2003).

While several studies have investigated the impacts of *Phragmites* on marsh community structure, less is known regarding trophic interactions with this invader. Several herbivores, such as insects, crustaceans, and gastropods, are known to feed on native marsh grasses and, in some cases, can strongly influence the distribution and abundance of these species (Silliman and Bertness, 2002; Pennings and Silliman, 2005; Silliman et al., 2005; Holdredge et al., 2009). Few studies have investigated whether native marsh grazers feed on *Phragmites*. Gratton and Denno (2006) demonstrated that in *Phragmites*-dominated marshes, arthropods obtained most of their basal nutritional resources from benthic microalgae and detritus, and not directly from *Phragmites*. When these marshes were restored to *Spartina*-domination, arthropods contained the isotope signatures of *Spartina* feeding. Thus, it appears that *Phragmites* invasion may alter trophic interactions among organisms in tidal marshes, and may not provide a functional replacement of *Spartina* as a basal resource for all consumers. Given that biological control by herbivores has been suggested as a method of controlling *Phragmites* (Tewksbury et al., 2002), it is important to investigate the interaction between *Phragmites* and native marsh herbivores.

One common grazer in mesohaline and polyhaline marshes is the periwinkle marsh snail, *Littoraria irrorata*, which consumes *Spartina*, but also uses it as a substrate for growing a fungus, on which they feed (Newel and Barlocher, 1993; Graca et al., 2000; Silliman and Newell, 2003). This snail has been shown to limit the abundance of *Spartina*, especially when abundance of this grazer is high (Silliman and Bertness, 2002; Silliman et al., 2005). In southern latitudes, grazing by snails (*L. irrorata*) and planthoppers can induce a chemical defense in *Spartina*, which deters feeding by snails (Long et al., 2011).

Currently, much of the mesohaline region of the Chesapeake Bay salt marsh is now dominated by *Phragmites* (Rice et al., 2000) and *L. irrorata* are observed climbing on stalks of this grass (L. Hendricks, H. Mossop, and C. Kicklighter, pers. obs.). As a first step toward examining feeding preferences of grazers for this invader in comparison to the native grass, *Spartina*, we compared the toughness of *Phragmites* to that of *Spartina* and conducted *L. irrorata* feeding experiments on these two plants. In addition, *Phragmites'* chemical deterrents against *L. irrorata* grazing were examined.

Methods and Materials

Experimental Organisms Periwinkle marsh snails (*Littoraria irrorata*), *Spartina alterniflora*, *Phragmites australis*, and the green seaweed *Ulva lactuca* all occur together in mesohaline marsh areas in the Chesapeake Bay and were collected May–July 2008 from the same site on the grounds of the Chesapeake Bay Environmental Center in Grasonville, MD, USA (N38°57.386′ W076°14.206′). *Phragmites* was confirmed to be exotic by restriction fragment-length polymorphism (RFLP) analysis of non-coding chloroplast DNA regions, following the methods of Saltonstall (2003) (L. Hendricks unpub. data). Grass stalks were clipped just above the sediment surface with shears and placed in large plastic bags. Snails and *U. lactuca* were collected by hand and placed in separate plastic bags. The snails were maintained in 38-l tanks with approximately 1 l of 15 ppt seawater (Instant Ocean, Atlanta, GA, USA) and were fed once a week with the control food (*U. lactuca*). *Phragmites* and *Spartina* samples for toughness measurements were used within 3 hr of collection. All other grass samples were frozen until used.

Toughness Previous studies have found that plant toughness can deter consumption by herbivores, so we measured toughness in two ways (based on the methods of Pennings et al., 1998): the force necessary to tear a leaf blade and penetrate a leaf blade. These methods likely replicate the actions of piercing and chewing grazers, but it is likely that neither technique exactly mimicks the piercing and scraping action of the snail radula, but may provide an indication as to which plant might be more difficult for snails to consume. To measure tearing, a safety pin pierced the center of a 5 cm long leaf blade, about 6 mm from the bottom of the blade, and fastened. The leaf blade was oriented vertically, and the top was held with two fingers while a plastic cup, which was attached to the safety pin via a string, was slowly filled with sand until the safety pin tore through to the end of the leaf blade. After this occurred, the safety pin + string + cup + sand were weighed.

Leaf penetration was measured with a plastic cup with a dull pin point sticking out of the center of the bottom. The pin was placed so that it rested in the middle of a 5 cm long leaf blade lying on a piece of wood, such that there was a

depression in the wood underneath where the pin point rested on the leaf. The cup was slowly filled with sand until the pin penetrated the leaf blade. When this occurred, the cup + pin + sand were weighed.

For both toughness measurements, one leaf from each of 15 plants was tested. Because the data were normally distributed (Shapiro Wilk and D'Agostino and Pearson Omnibus normality tests) and had similar variances (Bartlett's test) (Zar, 1999), we examined toughness differences between the plants with an unpaired *t*-test using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Feeding Bioassays Initial assays offering pieces of fresh plant tissues to *Littoraria irrorata* were unsuccessful, as the snails fed so slowly that tissues wilted before significant feeding differences could be detected. Because we were concerned that wilted tissues might differ in palatability from fresh, intact tissues, we conducted assays used dried, ground plant tissues, based on a combination of methods in Bolser and Hay (1996) and Kicklighter and Hay (2006). Briefly, reconstituted foods were created by lyophilizing and then grinding plant tissues with a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) into a fine powder. This was mixed with 0.03 $g ml^{-1}$ alginic acid, and distilled water was added to reconstitute plant tissues to a natural volume.

Three snails per replicate were offered equal amounts of ground *Spartina* and *Phragmites*, spread on opposite ends of window screen mesh strips. Snails fed until approximately half of one treatment was consumed (or for 24–48 h). Preference was determined by counting the number of squares of each food type consumed. Because samples were not normally distributed (as determined by Shapiro Wilk and D'Agostino and Pearson Omnibus normality tests) and variances were unequal (Bartlett's test), we analyzed food consumption with a Wilcoxon Matched Pairs Test ($N=12$ replicates) (Zar, 1999) using GraphPad Prism software.

To assess if *Phragmites* chemicals were responsible for feeding deterrence, a volumetric equivalent of *Phragmites* crude chemical extract or chemical fraction (see methods below) was resolubilized and added to a palatable control food (the seaweed *Ulva lactuca*) at a natural concentration found in the grass (as determined by volume). Three snails per replicate were offered equal amounts of the control food containing *Phragmites* chemicals vs. the control food without any chemicals added, as described above ($N=14$–31 replicates). Because samples were not normally distributed and variances were unequal (determined as described above), we analyzed food consumption with a Wilcoxon Matched Pairs Test (Zar, 1999) using GraphPad Prism software.

Crude Chemical Extraction and Separation Chemicals were extracted from *Phragmites* leaves, similar to Kicklighter et al. (2003). This method was chosen because it effectively extracts chemical defense compounds and avoids the use of dichloromethane, which can cause deterrent chemicals to degrade (Kicklighter and Hay, 2006). Leaves were cut up in approximately twice the volume of 100% methanol and 100% ethyl acetate, consecutively, for 2 hr each. Extracts were combined, filtered to remove solids, and the solvent removed with a rotary evaporator. This crude extract was combined with the control food at a natural concentration found in the grass and tested in a bioassay (as described above). To accomplish this, a volumetric equivalent of extract was resolubilized in methanol or ethyl acetate and transferred to a 20 ml scintillation vial. The solvent was removed by drying the extract under a stream of air. Previous assays demonstrated that the solvent carrier did not deter snail feeding, so any decreases in snail feeding on foods treated with *Phragmites'* chemicals should be attributed to the chemicals, and not to the solvent. Thus, control foods were not treated with the carrier solvent. Because the crude extract deterred snail feeding (as determined by a Wilcoxon Matched Pairs Test, $N=12$), we performed a modified inverse Kupchan solvent-solvent partition (Kupchan et al., 1975). Our one modification to this partitioning scheme was to omit the dichloromethane/water partition, since previous studies have found dichloromethane to degrade the activity of deterrent compounds (Kicklighter and Hay, 2006). This resulted in partitioning *Phragmites* chemicals into four fractions that differed in solubility: those chemicals that were soluble in hexanes, ethyl acetate, butanol, and water. Each fraction was tested in the bioassay against the palatable control food and feeding statistically analyzed, as described above. The deterrent fractions (butanol and ethyl acetate) were further separated by C18 reversed phase column chromatography. Mobile phases were progressive dilutions of water and methanol—100% water, 75:25 water:methanol, 50:50 water:methanol, 25:75 water:methanol, and 100% methanol (and a final wash of ethyl acetate which was added to this fraction). Subsequent active fractions were separated further by reversed phase chromatography and preparative thin layer chromatography (TLC), but repeated attempts resulted in loss of activity and further separation was not achieved.

Phenolic and Protein Assays Previous studies have suggested that phenolic compounds are responsible for deterring herbivores from feeding on plants, including marsh grasses (Coley et al., 1985; Herms and Mattson, 1992; Barlocher and Newell, 1994). In addition, *Phragmites* has been shown to contain these compounds (Rudrappa and Bias, 2008). Following the methods of Siska et al. (2002), who examined phenolic content of

marsh grasses, we measured phenolic concentrations using a Folin-Dennis reaction in our *Phragmites* butanol- and ethyl acetate-soluble fractions, and reversed phase column fractions of the ethyl acetate fraction. Aliquots of plant chemicals representing 0.1 ml of plant tissue were dissolved in 50 µl of 80% methanol and reacted with 750 µl deionized H_2O, 200 µl Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, MO, USA), and 1000 µl 20% Na_2CO_3. After a 20-min incubation period, absorbance was read at 700 nm on a Thermo Electron Biomate 3 spectrophotometer (Thermo Electron Corp, Waltham, MA, USA). A standard curve was constructed using ferulic acid (Siska et al., 2002).

Because low nutritional quality can interact with chemical defenses to make them more potent (e.g., Hay et al., 1994), *Phragmites* and *Ulva lactuca* tissues were analyzed for protein content, using a standard Bradford assay (Bradford, 1976). Because *U. lactuca* contained less protein than *Phragmites*, we wanted to make sure that the lower nutritional quality of *U. lactuca* was not enhancing the deterrence of *Phragmites'* chemical defense and confounding our results. Thus, we performed a bioassay (as described above), where we added protein albumin (bovine serum albumin, Sigma-Aldrich, St. Louis, MO, USA) to the ground *U. lactuca* used to make the treatment (*Phragmites* crude extract added) and control foods to increase the protein concentration to a level comparable to *Phragmites* tissue. Because samples were not normally distributed and variances were unequal, we analyzed food consumption with a Wilcoxon Matched Pairs Test.

Results

When we compared the toughness of *Phragmites* to *Spartina*, we found that the force required to either tear through (Fig. 1a) or pierce (Fig. 1b) *Spartina* was 1.3X that of *Phragmites*. When plants were ground up into a fine power and reconstituted into a gelled food, however, *Littoraria irrorata* consumed 26X more *Spartina* than *Phragmites* (mean ± S.E.M=76.7%±4.7 vs. 2.9% ±2.0, respectively; Wilcoxon Matched Pairs Test, $P<0.001$, $N=12$).

The *Phragmites* crude extract strongly deterred snail feeding (Fig. 2), suggesting that a chemical deterrent best explains snail feeding preferences. When a *Phragmites* crude extract was fractioned using an inverse Kupchan partition, the water- and hexanes-soluble chemicals were not significantly deterrent, while both the butanol and ethyl acetate fractions deterred feeding (Fig. 3a). Both actively deterrent fractions were separated further by reversed phase chromatography. Of the five reversed phase column fractions from the ethyl acetate-soluble fraction, only the 50:50 water:methanol fraction actively deterred feeding

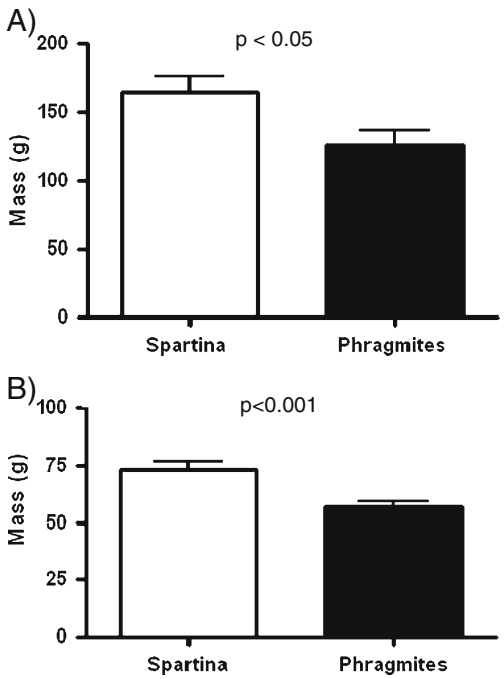


Fig. 1 Toughness of leaves of *Phragmites* and *Spartina* as measured by **a** tearing; and **b** penetration. $N=15$ leaves, analyzed with an unpaired *t*-test. Error bars represent standard error of the mean

(Fig. 3b). Similarly, only the 50:50 water:methanol fraction of the butanol reversed phase fractionation was deterrent (Fig. 3c).

We attempted further separation of the 50:50 water:methanol fraction from the butanol and ethyl acetate partitions by preparative thin layer chromatography and reversed phase column chromatography, but activity was repeatedly lost in the resulting fractions. Even when all the fractions of subsequent separation efforts were added back together at three times natural plant concentrations, the chemicals were not deterrent to feeding, suggesting that the active molecule(s) decomposed. Other separation techniques (e.g., LH-20 size exclusion chromatography, anion-exchange chromatography) were not attempted.

Because many plants are thought to utilize phenolic compounds as chemical defenses (Coley et al., 1985; Herms and Mattson, 1992; Barlocher and Newell, 1994), phenolic content was measured for the butanol and ethyl acetate fractions resulting from the Kupchan partition (Fig. 3a). Their concentrations were similar, with phenolic levels in both partitions being 0.12% of dry mass. Phenolic concentrations also were measured in all fractions in the reversed phase column separation of the ethyl acetate partition. Measured concentrations (as % of dry mass) were as follows: 0.001% in the 100% water fraction, 0.02% in 75:25 water:methanol, 0.03% in 50:50 water:methanol, 0.04% in 25:75 water:methanol, 0.03% in 100% methanol.

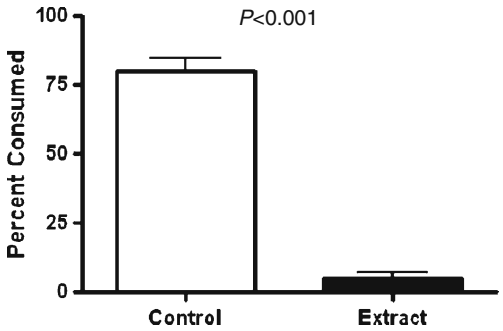


Fig. 2 Snail feeding on dried, ground, reconstituted *Ulva lactuca* lacking (*clear bar*) or mixed with (*black bar*) *Phragmites* crude extract. $N=14$ replicates, analyzed with a Wilcoxon Matched Pairs test. Error bars represent standard error of the mean

The protein content of ground *Phragmites* and *Ulva lactuca* were measured by Bradford assay. *Phragmites* had a protein content of approximately 0.0432 g protein ml^{-1}, while *Ulva* had approximately 0.0063 g protein ml^{-1}. Thus, *Phragmites* contains 6.9 times more protein than *U. lactuca*. When protein was added to *U. lactuca* to achieve the protein concentration of *Phragmites* and the crude extract feeding assay was retested, *Littoraria irrorata* still significantly preferred *U. lactuca* lacking the *Phragmites* crude extract (mean ± S.E.M.=76.1%±5.4 vs. 25.7%±8.9, Wilcoxon Matched Pairs Test, $P<0.001$, $N=14$). Thus, low nutritional value of our gelled food likely did not influence snail feeding.

Discussion

Mesohaline regions of the Chesapeake Bay are shifting from marshes dominated by *Spartina* to ones containing a large abundance of the invasive grass *Phragmites* (Rice et al., 2000; Lathrop et al., 2003; King et al., 2007). This may have important implications for marsh community structure through alteration of trophic interactions. When performing feeding assays using *Phragmites'* chemical extracts, snail feeding was depressed (Figs. 2 and 3), suggesting *Phragmites* is chemically defended against this grazer. *Spartina* was tougher to tear and penetrate than *Phragmites* (Fig. 1), however, the periwinkle marsh snail *Littoraria* preferred *Spartina* over *Phragmites* when offered reconstituted,

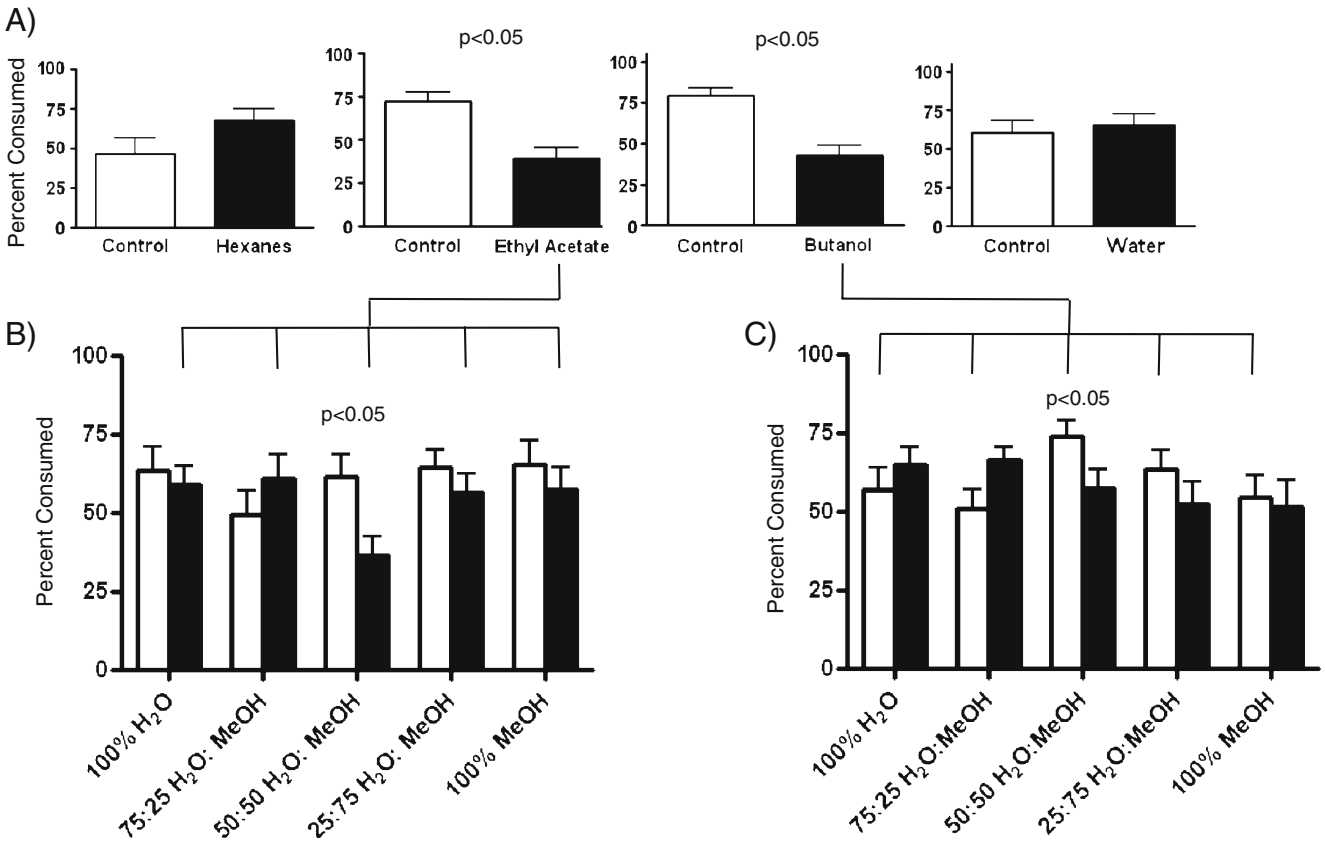


Fig. 3 Bioassay guided fractionation of the deterrent components in the *Phragmites* crude extract based on snail feeding deterrence. Consumption of control food vs. food mixed with *Phragmites* chemicals resulting from **a** solvent-solvent partition; **b** fractions produced by the reversed phase separation of the ethyl acetate partition produced in (**a**); and **c**) fractions produced by the reversed phase separation of the butanol partition produced in (**a**). $N=15–31$ replicates, data and analysis, and error bars as described in Fig. 2

ground tissues of these two plants. Ground tissues lacked the structural attributes of the natural plants, so the fact that the snails preferred *Spartina*, the tougher plant, suggests that plant structural attributes may be less important in determining snail feeding, which is similar to findings of Siska's et al. (2002) survey of salt marsh plants. Feeding assays with fresh, intact plants are required, however, to more fully understand mechanisms other than chemical defenses that may mediate snail feeding preferences.

Phragmites contained 6.9x the protein content as our palatable food (and 1.3x the protein of *Spartina*, L. Hendricks unpub. data), and we, therefore, excluded the possibility that *Phragmites* was eaten less than other plants due to poor nutritional quality. Previous studies have shown low nutritional quality to minimize herbivory (Cruz-Rivera and Hay, 2003). To account for low nutrition in our palatable control food, snails were offered a low-quality food choice with the chemical defense of the more nutritious plant, *Phragmites*. Snails may have avoided *U. lactuca* when it contained *Phragmites* chemicals because the food was simply too low in nutrition to be worth consuming deterrent chemicals. However, when protein albumin was added to *U. lactuca*, so that the food matched the protein concentration of *Phragmites*, the crude extract feeding assay yielded the same results: *Phragmites* chemicals deterred feeding. Thus, nutritional content did not appear to confound the results of the extract feeding assays.

Both the ethyl acetate and butanol partitions of the *Phragmites* crude extract deterred feeding (Fig. 3a), initially suggesting more than one deterrent compound. Further separation of both partitions, however, yielded active fractions of the same polarity (molecules soluble in a 50:50 water:methanol mixture) (Fig. 3b and c). Given this similarity and the fact that both the ethyl acetate- and butanol-derived 50:50 water:methanol fractions appeared similar by thin layer chromatography, we assumed that our deterrent compound(s) initially separated into both the butanol and ethyl acetate fractions and that the deterrent molecule(s) present in both 50:50 water:methanol fractions are the same. Because the active fraction contains molecules soluble in 50:50 water:methanol, they are moderately polar. This is similar to the findings of Siska et al. (2002) who demonstrated that polar fractions of some common marsh plants (*Aster tenuifolius*, *Iva frutescens*, *Spartina alterniflora*, and *S. patens*) from southern U.S. Atlantic regions deterred feeding by a crab. Long et al. (2011) also found a polar extract from *Spartina* that had induced a chemical defense to deter feeding by *L. irrorata*. Since our separation methods differed from the above studies, however, it cannot be determined whether *Phragmites*' deterrent compounds are the same or similar as those tested by Siska et al. (2002) and Long et al. (2011).

While our findings suggest that *Phragmites* is chemically defended, we were unable to isolate and identify the chemical(s) mediating its resistance against snail herbivory, due to repeated loss of deterrence of our chemical fractions. Even when all of these chemical fractions were recombined and used in a feeding assay at three times natural plant concentrations, there was no feeding deterrence, indicating that the deterrent compound(s) decomposed as a result of the separation process.

In an effort to gain some understanding of the molecules mediating *Phragmites'* chemical defense, we quantified phenolic concentrations in some of our *Phragmites* extracts. Several studies have suggested that phenolics are important chemical defenses for terrestrial plants, including *Spartina alterniflora*, and these compounds can be unstable (Barlocher and Newell, 1994; Herms and Mattson, 1992; Coley et al., 1985). In addition, *Phragmites* has been shown to contain phenolics (Rudrappa and Bias, 2008), thus, we examined *Phragmites* for these metabolites. We did detect phenolics in all samples tested, but at fairly low levels. The deterrent ethyl acetate and butanol partitions each only contained a total phenolics concentration of 0.12% dry mass, and fractions resulting from the reversed phase separation of the ethyl acetate partition had phenolics concentrations that ranged from 0.001% to 0.04% dry mass. In addition, a non-deterrent fraction (25:75 water:methanol) contained more phenolics than the deterrent fraction (50:50 water:methanol), suggesting that total phenolics do not play a role in *Phragmites'* defense. As a comparison, Siska et al. (2002) found phenolic concentrations in *Spartina alterniflora* leaves to be between 2.45% and 3.17% dry mass and it was the plants that contained the higher concentrations of phenolics that were less palatable to a crab and two insects. Based on our data, it is impossible to know whether phenolics mediate *Phragmites'* chemical defense against snails, but it seems unlikely that the total phenolics concentration is high enough for this function. It is possible, however, that a single phenolic compound, found in a low concentration in the 50:50 water:methanol fraction does mediate deterrence. Further studies are needed to confirm or refute this.

Gallic acid is secreted by the invasive form of *Phragmites* and has been shown to inhibit the growth of other plants (Rudrappa et al., 2007). It is possible that gallic acid also serves as a chemical defense for *Phragmites*, deterring snail feeding. Gallic acid is polar [our compound (s) is moderately polar], however, it displays thin layer chromatography characteristics different from our deterrent fraction. Thus, it is unlikely that it plays a defensive role against this herbivore.

Invasion of marshes by *Phragmites* may alter trophic interactions, resulting in disruption of the marsh community. If *Littoraria irrorata* preferentially consume *Spartina* and

use it for a substrate for growing fungus (Silliman and Newell, 2003) over *Phragmites*, this could facilitate the decline of *Spartina* and the spread of *Phragmites* in marshes where *L. irrorata* are abundant. Fewer fungal spores are found on *Phragmites* compared to *Spartina* plants in the same area (H. Mossop, unpub. data), suggesting that snails may not use *Phragmites* for growing fungus. Similarly, some insect herbivores (such as aphids and gall flies) preferentially consume native *Phragmites* over introduced *Phragmites*, which can result in new shoot death (Lambert, 2005), stunted growth (Park and Blossey, 2008), and decreased flowering (Lambert et al., 2007) in the native form. In areas dominated by *Phragmites* and devoid of *Spartina*, snails, and possibly other herbivores, may switch to alternate food sources, as has been shown for arthropod consumers by Gratton and Denno (2006), or decline in abundance. Further studies are required to more fully understand the interactions between *Phragmites*, herbivores, and *Spartina* and how they may impact marsh ecosystems.

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Mass Spectrometry Based Metabolomics to Identify Potential Biomarkers for Resistance in Barley against Fusarium Head Blight (*Fusarium graminearum*)

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Abstract Resistance in Triticeae to fusarium head blight (FHB) is quantitatively inherited. Metabolomics as a tool was used to better understand the mechanisms of resistance and to identify potential FHB resistance biomarker metabolites in barley. Five FHB-resistant two-row barley genotypes (CIho 4196, Zhedar-1, Zhedar-2, Fredrickson, and Harbin-2r) and one FHB-susceptible genotype (CH 9520–30) were each inoculated with either pathogen-suspension or mock-solution. Disease severity, quantified as the proportion of spikelets diseased, varied among genotypes, being the greatest in CH 9520–30. Spikelets were sampled, metabolites extracted with aqueous methanol, and analyzed using an LC-ESI-LTQ-Orbitrap system. A pair wise, resistant vs. susceptible, *t*-test identified 1774 significant treatment peaks. Canonical discriminant analysis of peak abundance allowed the genotypes to be sorted into three clusters: (i) CH9520-30, (ii) Harbin-2r, (iii) the remaining four genotypes. The *t*-test was further used to identify resistance-related (RR) and pathogenesis-related (PR) metabolites. The pathogen-produced virulence factor deoxynivalenol (DON), and its detoxification product, DON-3-*O*-glucoside (D3G) were designated as resistance indicator (RI) metabolites. Metabolites (RR, PR, or RI) occurring in at least two resistant genotypes, showing a two-fold or greater abundance in resistant vs. susceptible lines, and also known to have plant defense functions were selected as potential FHB resistance biomarker metabolites. These included phenylalanine, *p*-coumaric acid, jasmonate, linolenic acid, total DON produced (TDP), and the proportion of DON converted to D3G (PDC). Total DON was the lowest in CIho 4196, while PDC was the highest in Zhedar-2. The application of RR, PR, and RI metabolites as potential biomarkers to enhance resistance is discussed.

Key Words *Fusarium graminearum* · *Gibberella zeae* · Biomarkers · Metabolomics · Functional genomics · Barley · FHB · DON

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Introduction

Fusarium head blight (FHB) is a destructive disease of barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.), with a worldwide economic impact (Gilbert and Tekauz, 2000). The disease is caused by several *Fusarium* species of which *F. graminearum* (teleomorph: *Gibberella zeae*) is of greatest relevance. *Fusarium graminearum* significantly reduces both grain yield and quality by producing trichothecene mycotoxins, such as deoxynivalenol (DON) (Desjardins 2006). Genetic improvement of host resistance to

FHB is considered the best disease management option to reduce mycotoxin production and mitigate yield loss.

Plant resistance mechanisms to pathogen attack can be classified as apparent or true (Agrios, 2005). Apparent resistance is not directly controlled through a genomic influence on metabolism, but rather the plant escapes disease. For example, the closed-floret two-row barley genotypes are more resistant to infection than open-floret six-row genotypes (Bai and Shaner, 2004; Tekauz et al., 2000). True resistance is genetically controlled, and the mechanisms are either structural or biochemical, where the latter can be associated with particular proteins or metabolites. Quantitative FHB resistance documented in wheat and barley is associated with resistances to spikelet infection (Type I), spreading of the infection within the spike (Type II), and limiting trichothecene accumulation (Type III), especially DON, following pathogen invasion (Miller et al., 1986). While these categories of resistance have served in genotypic screening, they do not speak to the issue of resistance mechanisms. As mutants of *F. graminearum* lacking DON/trichothecene production fail to spread within infected spikes of wheat, DON is clearly an important virulence factor (Proctor et al., 1995; Jansen et al., 2005). In general, barley exhibits high type II resistance, therefore the focus of breeding programs has been on improving type I resistance (Bai and Shaner, 2004). However, ranking of barley genotypes based on type I resistance has proven to be inconsistent over years and seasons. This variation generally has been attributed to environmental conditions. More than 100 FHB-resistance quantitative trait loci (QTL) have been identified, across all seven chromosomes of wheat and barley, but several of these are either unstable or often associated with pleiotropic effects (Choo, 2006; Buerstmayr et al., 2009). The mechanisms of true resistance have been partially elucidated for a single QTL (chromosome 3BS), which confers a high rate of conversion of DON to DON-3-*O*-Glucoside (D3G) in resistant lines through the enzymatic action of DON-glucosyltransferase (Lemmens et al., 2005). The identification of FHB resistance mechanisms in barley would not only explain the functions of over 100 known FHB resistance QTLs but also would provide molecular breeders with novel tools for selecting resistant genotypes. In the present study, barley genotypes commonly used as sources of resistance in breeding and in QTL mapping programs were selected to explore and provide an overview of FHB resistance mechanisms controlled by metabolites.

Metabolomics technology has been applied to identify resistance-related (RR, greater abundance in resistant than in susceptible genotype) metabolites in wheat and barley. Based on gas chromatography and mass spectrometry (GC-MS), several RR metabolites have been identified (Hamzehzarghani et al., 2008). However, metabolomics based on GC-MS detect only volatile metabolites (Vorst et al., 2005). Based on LC-hybrid-MS (LC-ESI-LTQ-Orbitrap), more than 3000 peaks have been detected, and several RR metabolites have been identified in spikelets of barley in response to FHB infection (Bollina et al., 2010; Kumaraswamy et al., 2011). The metabolites identified belonged to three main chemical groups: phenylpropanoids, fatty acids, and terpenoids. While prior studies were based on an analysis of paired resistant/susceptible genotypes, in this study, the occurrence of RR metabolites was investigated in more than one resistant genotype, using an LC-MS method.

Methods and Materials

Plant Production Five FHB-resistant two-row barley genotypes (CIho 4196, Fredrickson, Harbin-2r, Zhedar-1, and Zhedar-2) and one FHB-susceptible genotype (CH 9520–30) were used. Three seeds of each genotype were sown in individual 15 cm pots and maintained under greenhouse conditions at 22±3°C. Plants were irrigated daily and fertilized at 2 wk intervals with 200 ml of 0.3% PlantProd (20-20-20 NPK + trace elements). Following germination, only the main stem and a single tiller of each plant were retained. These genotypes, along with several others, also were sown in a field in Ottawa, Ontario, Canada, as detailed in Choo et al. (2004). For each genotype, 10 g of seeds were sown in two rows of 1.5 m in length.

Pathogen Production, Inoculation, and Incubation Fusarium graminearum Schwabe isolate 15–35 was maintained on potato dextrose agar media, and fresh cultures were produced on rye agar media. Seven-d old cultures were used to collect macroconidia. Barley spikelets were spray inoculated with 1×10^5 macroconidia ml^{-1} or an aqueous solution containing 0.02% Tween80 (mock), at 50% anthesis to early milky stage (GS=65–73; Zadoks et al., 1974). The inoculated plants were placed inside plastic bags the inner surfaces of which had been sprayed with sterile water. The plants were maintained under these conditions for 48 h in order to facilitate initial establishment of the infection.

Experimental Design and Statistical Analyses The greenhouse experiment was designed as a randomized complete block, with twelve treatments, consisting of six genotypes and two inoculations of mock-solution (M=0.02% Tween80) or pathogen (P = macroconidia in 0.02% Tween80), with five replicates in time, conducted at roughly weekly intervals. Each experimental unit consisted of a pooled sample of 50–60 spikelets, collected from 6 plants. The field experiment was designed as a randomized complete block with four replicates (Choo et al., 2004).

The experimental units consisted of 30 spikes per replicate. The resistance of the different genotypes was confirmed by assessing the proportion of spikelets diseased (PSD). The PSD was determined from the number of diseased spikelets in spray inoculated spikes 21 d after inoculation in the greenhouse or 1 mo after inoculation in the field.

Sampling and Metabolite Extraction Some 50–60 inoculated spikelets, from 6 spikes, were harvested 72 h after inoculation. The lemmas and paleas were removed and frozen in liquid nitrogen, and metabolites were extracted (Kumaraswamy et al., 2011). In brief, the samples were crushed in liquid nitrogen with a mortar and pestle. A finely powdered 100 mg (fresh weight) sample was placed in a 2.2 ml micro-centrifuge tube, to which 1400 µl of ice-cold methanol (HPLC grade 99.96% pure, Fisher Scientific), 200 pg of genistein, and 200 pg of ribitol as internal standards, and 1,500 µl of water were added. After vortex mixing for 10 sec, the samples were sonicated for 15 min at a frequency of 40 kHz in a water bath at room temperature. The tubes then were centrifuged for 10 min at 20,000 g. The supernatants were filtered through a 0.1 µm PVDF membrane filter (Millipore Corporation, Bedford, MA, USA), and the filtrate was collected and stored at −20°C, and analyzed within a week.

Metabolite Analysis Using LC-ESI-LTQ-Orbitrap Metabolites were analyzed using an LC-hybrid-MS system (LC-ESI-LTQ Orbitrap, Thermo Fisher, Waltham, MA, USA). Semipolar metabolites were analyzed with a capillary reverse phase C-18 column (500 µm i.d. × 10 cm) packed with Jupiter 5 µm particle 300 Å reverse-phase stationary material (Phenomenex, Torrance) (Kumaraswamy et al., 2011). Polar metabolites, mainly trichothecenes from pathogen inoculated samples, were analyzed with a polar column, C18 Kinetex (210 µm i.d×10 cm, with a particle size of 2.6 µm, Phenomenex). The column was maintained at 25°C, and the mobile phase was adjusted to a flow rate of 150 µl min^{-1} and eluted with water containing 2.5 mM ammonium acetate (A) and methanol (B). A gradient elution profile was used starting with 98% mobile phase A for 4 min, then changing to 10% mobile phase A over 26 min, and subsequently ramped to 90% mobile phase B over 5 min, followed by a 5 min ramp to 100% mobile phase B, and a rapid return to 100% mobile phase A. A blank sample was run after every experimental sample to reduce carry over. Some samples were analyzed in MS/MS mode, using 35 eV CID energy, to determine the fragmentation patterns. The compounds DON, 3ADON, and DON-3-*O*-glucoside (D3G) (Sigma-Aldrich, Oakville, ON, Canada) were spiked in different concentrations, using the above LC-hybrid-MS protocol. The abundances of DON, 3ADON, and D3G, rather than their acetate adducts, were used to develop regression models to predict their concentrations from abundances.

LC-MS Output Analysis Raw data obtained from LTQ Orbitrap, based on semipolar and polar columns, were separately aligned using XCMS 1.22.1 (Kumaraswamy et al., 2011). Data were processed with a signal to noise threshold of, *snthresh*=5:1 and band width of, *bw*=10 s. The CAMERA bioinformatics tool (Tautenhahn et al., 2007) was used to annotate peaks with isotopes, neutral losses, adducts, and dimmers, which represented multiple peaks of the same compound. The data on peak abundance (relative ion counts) were subjected to a student's *t*-test using SAS (version 9.2; SAS Institute Inc., Cary, NC, USA) to select metabolites that had significant treatment effects. The tests of hypotheses included four treatment combinations: RM vs. SM, RP vs. RM, SP vs. SM, RP vs. SP, where R = resistant, S = susceptible, M = mock, P = pathogen. The peaks with significant ($P<0.05$) treatment effects were designated as metabolites.

The log transformed abundances of treatment significant metabolites, from mock-inoculated and pathogen-inoculated, were separately (because of the complex design) subjected to canonical discriminant analysis (CDA), to group observations, and identify the metabolites significantly loading to a group of treatments. The treatment significant metabolites also were further used to select pathogenesis related (PR), resistance related (RR), and resistance indicator (RI) metabolites. A metabolite with significantly higher abundance in a pathogen-inoculated than in a mock-inoculated genotype was considered a PR metabolite, be it in resistant (PRr=RP>RM) or susceptible (PRs=SP>SM) genotypes. A metabolite with a higher abundance in the resistant genotype than in a susceptible one was considered an RR metabolite. An RR metabolite based on mock-inoculation was considered as a constitutive (RRC= RM>SM) metabolite. A PRr metabolite in a resistant genotype with abundance greater than that in the susceptible pathogen-inoculated was considered as RR induced (RRI= RP>RM and RP>SP) metabolite (Hamzehzarghani et al., 2008). A relative reduction in the accumulation of the virulence factor DON (Proctor et al., 1995) in a resistant vs. susceptible plant served to indirectly assess two different plant resistance mechanisms: i) the reduction in DON synthesis by the pathogen, possibly due to high abundance of phenolics and flavonoids in the resistant genotype (Boutigny et al., 2009; Kushalappa lab unpublished data); or ii) the increased detoxification or conversion of DON to D3G, through the enzyme DON-glucosyltransferase (Lemmens et al., 2005; Bollina et al., 2010). The concentration of total DON produced was calculated as TDP = DON + D3G, using the regression models developed here to calculate concentrations from abundances. The proportion of TDP

converted to D3G was calculated as: PDC = D3G/TDP. Both TDP and PDC were designated as resistance indicator (RI) metabolites. The PR, RR, and RI metabolites were assigned with putative names, based on accurate mass, fragmentation pattern that also considered manual verification, and isotope ratio, either matching with the spectra in databases or those obtained by spiking authentic standards (Bollina et al., 2010; Kumaraswamy et al., 2011).

Results

Disease Severity FHB severity (PSD), under greenhouse conditions, ranged from 0.54 in the susceptible genotype (CH 9520–30) to 0.17 in the most resistant genotype (CIho 4196). The PSD was not significantly different among five resistant genotypes tested, but was significantly lower in all resistant genotypes compared to the susceptible (Fig. 1). In field evaluations, the susceptible genotype CH 9520–30 had PSD of 0.48 as compared to 0.11 for CIho 4196. The ranking of six genotypes based on PSD in greenhouse evaluations were similar to those under field evaluations, except for Zhedar-1, which had higher PSD under field conditions.

Multivariate Analysis of Metabolic Profiles A total of 1774 metabolites had significant treatment effects. The abundances of the treatment-significant peaks from pathogen and mock-inoculated samples were separately subjected to canonical discriminant analysis (CDA) to better visualize the grouping of observations and genotypes. In both, the five replicates of different treatments were closely grouped, meaning there was little experimental error. In mock-inoculation (Fig. 2a), the CAN1 vector explained 60% of variance, with four resistant genotypes grouped together with high positive scores, and the fifth resistant genotype Harbin-2r as well as the susceptible genotype CH9520-30 both having negative scores. The genotypes CIho 4196 and Fredrickson had the highest positive scores. The metabolites with high positive loading to CAN1 vectors were: caffeoyl alcohol, kaempferol 7,4′-dirhamnoside, nallaflavanone, kaempferol 3-rhamnoside, linoleic acid, *p*-coumaric acid, and phenylalanine (Table S1a). In pathogen inoculated treatments (Fig. 2b), the CAN1 explained 41% of variance, where the resistant genotypes had high positive CAN1 scores, while the susceptible one had a highly negative CAN1 score. Although not all the high loading (L>0.9) metabolites were identified, among those that were, octadecanoic and *p*-coumaric acids had relatively high loadings (Table S1b).

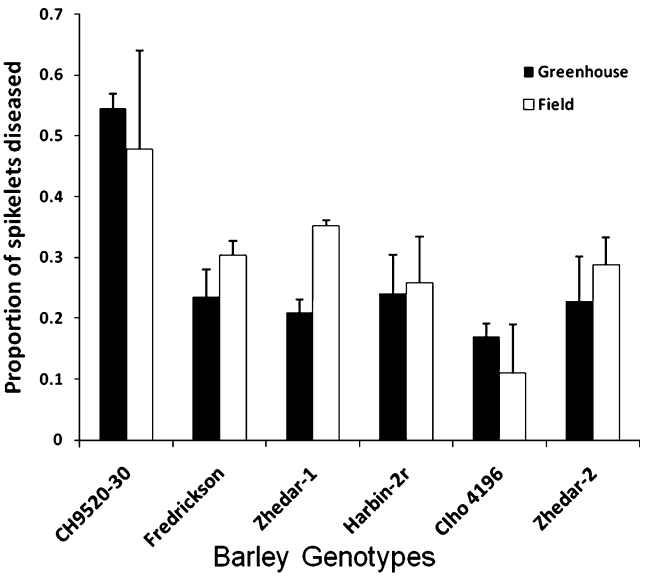


Fig. 1 Proportion of spikelets diseased in a spike (PSD), in six barley genotypes under greenhouse and field tests

Resistance Related Constitutive Metabolites (RRC) In total, 108, 84, 76, 106, and 69 RRC metabolites were detected in the genotypes CIho 4196, Zhedar-1, Zhedar-2, Fredrickson, and Harbin-2r, respectively (Table 1). Most of the RRC metabolites detected were phenylpropanoids. The magnitude of change in abundances of RRC metabolites relative to the susceptible variety varied among the resistant genotypes. A total of 14 RRC metabolites were common to two or more resistant genotypes, and their relative abundance was equal to or greater than two fold that in the susceptible genotype. The abundance of *p*-coumaric acid was 4.3 and 3.1 fold in Zhedar-2 and Fredrickson, respectively, while phenylalanine was nearly 2 fold in Zhedar-2, Zhedar-1, and Harbin-2r genotypes.

Pathogenesis Related (PR) and Resistance Related Induced Metabolites (RRI) The genotypes CIho 4196, Zhedar-1, Zhedar-2, Fredrickson, and Harbin-2r had 12, 11, 26, 14, and 13 significantly induced metabolites (PRr), respectively, following pathogen inoculation. However, the amount of induction in resistant genotypes was not significantly higher than in susceptible genotypes, meaning that none of these were RRI metabolites. Jasmonic acid was a PRr metabolite in all five resistant genotypes. Linolenic acid was common to two resistant genotypes and two fold greater in abundance than in the susceptible genotype.

Resistance Indicator Metabolites (RI) The extra cellular trichothecenes produced by the pathogen, DON, and 3ADON, and the plant enzymatic detoxification product of DON, D3G, were detected not only in their pure forms but also as their acetate (CH_3COO^-) adducts (Figs. S1, S2). The total amount of DON produced (TDP) by the pathogen, in lemma and palea, varied from 27 mg kg^{-1} in CIho 4196 to 53 mg kg^{-1} in Harbin-2r (Fig. 3a; Table 2). The amount

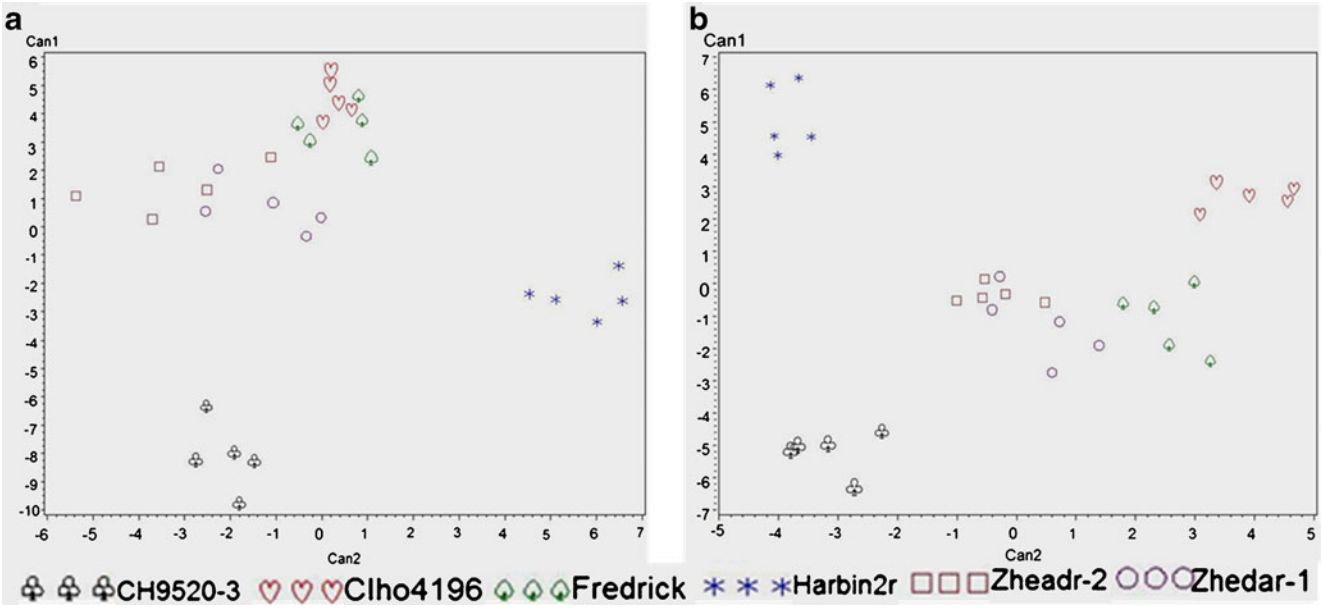


Fig. 2 Canonical discriminant analyses (CDA) of metabolic profiles of six barley genotypes: a = metabolic profiles of pathogen inoculated; b = metabolic profiles of mock-inoculated

of 3ADON, a precursor of DON, produced was quite low and it varied from 3.1 mg kg^{-1} in CH9520–30 to 4.4 mg kg^{-1} in Harbin-2r (Fig. 3a). The TDP was 50 mg kg^{-1} in susceptible genotypes CH9520–30, which was similar to the level in Harbin-2r. The PDC varied from 0.68 in CH9520–30 to 0.78 in Zhedar-2, and was moderate in Fredrickson, Harbin-2r, CIho 4196, Zhedar-1 (Fig. 3b; Table 2).

PR, RR and RI Metabolites in Resistant Genotypes

CIho 4196 The most resistant genotype CIho 4196 had 109 RRC and 12 PRr metabolites. Several of the RRC metabolites showed many fold (×) changes in abundance, especially for conjugates of kaempferol (e.g., 2.0–7.6 fold changes). The changes in abundance of other RRC metabolites were: *p*-Coumaric acid=2.8×, phenylalanine= 2.1×, linoleic acid=3.8×, JA=3.6×. Interestingly, the abundances of linoleic acid, linolenic acids, *p*-coumaric acid and phenylalanine increased more than two fold, following pathogen inoculation (PRr). The TDP was the lowest (27 mg kg^{-1}) and the PDC was moderate (0.70), among the genotypes tested in this study (Table 2).

Zhedar-1 Had 84 RRC and 11 PRr meabolites (Table 1). The highest fold changes in abundance of RRC metabolites were observed for traumatic acid=2.1×, linoleic acid=1.9×, phenylalanine =2.2×, and eucommin A (lignan)=3.2×. The changes for PRr metabolites were JA=2.3× and stearic acid= 2.1×. The TDP was the second lowest (33 mg kg^{-1}), but the PDC was quite low (0.70).

Zhedar-2 Had 76 RRC and 26 PRr metabolites. Large changes in abundance of RRC metabolites were observed for phenylalanine=2.4×, *p*-coumaric acid=4.3×, 4-geranyloxy-5-methylcoumarin=2.3×, and eucommin A (lignan)=1.4×, linolenic acid=3.7×, and oleic acid=1.9×. In addition, several flavonoids, as conjugates of kaempferol, were identified as RRC: 6-hydroxykaempferol 7,4′-dimethyl ether 3-sulfate (4.7×), kaempferol 7,4′-dirhamnoside (3.0×), kaempferide 5-glucoside-7-glucuronide (4.2×), kaempferol 3-(2″,3″-diacetyl-4″-*p*-coumarylrhamnoside (5.9×), and kaempferol 3-isorhamninoside (5.1×). The changes in abundance of PRr metabolites were: tuberculatin (lignan)= 1.3× and JA=3.0×. The TDP was moderate (37 mg kg^{-1}), but the PDC was the greatest among the genotypes evaluated (0.78) (Table 2).

Fredrickson This genotype had 106 RRC and 14 PRr metabolites. Several phenylpropanoids and fatty acids were identified as RRC metabolites (Table 1). The changes in abundance were: *p*-coumaric acid=3.1×, and an esterified coumaric acid, 4-geranyloxy-5-methyl coumarin=2.7×. Other RRC metabolites were caffeoyl alcohol, tuberculatin (a lignan), traumatic acid, and stearic acid. The PRr metabolites were JA, linolenic acid, and heptadecatrienoic acid.

Harbin-2r Had 69 RRC and 13 PRr metabolites. Among the ten RRC identified, four were phenylpropanoids and their change in abundance were: 4-geranyloxy-5-methyl coumarin=3.2×, phenylalanine =2.0×, calophyllolide= 2.3×, and podorhizol beta-D-glucoside (lignan)=1.7×. Among the four PRr metabolites identified, nortracheloge-

Table 1 Phenylpropanoid, flavonoid, and fatty acid RR-metabolites identified in five *Fusarium* head blight (FHB) resistant barley genotypes, as compared to susceptible CH 9520–30

Accurate Mass	Compound ID	CIho 4196	Zhedar-1	Zhedar-2[a]	Fredrick son	Harbin-2r
Phenylpropanoids						
164.0473	*p*-Coumaric acid	2.8*PRr		4.3***RRC	3.1*RRC	
165.0790	Phenylalanine	2.1**PRr	2.2*RRC	2.4**RRC		2.0**RRC
166.0629	Caffeyol alcohol				1.7*RRC	
294.1103	7-(2,3-Dihydroxy-3-methylbutyloxy)-5-hydroxy-5-methoxycoumarin					1.4*PRr
312.1725	4-Geranyloxy-5-methyl coumarin			2.3*RRC	2.7*RRC	3.2**RRC
374.1366	Nortrachelogenin					3.8**PRr
400.1158	4′-Demethylpodophyllotoxin	1.4*PRr				
416.1624	Calophyllolide	1.6*RRC	2.4*RRC		3.0***RRC	2.3*RRC
428.1682	*trans-p*-Ferulyl alcohol 4-O-[6-(2-methyl-3-hydroxypropionyl)] glucopyranoside	2.3*PRs				
488.1471	Bisosthenon	1.5*RRC				
640.1792	Chartreusin	1.7*RRC	1.7*RRC	1.8**RRC		
512.1319	Tuberculatin			1.7*PRr	1.6*RRC	
550.2050	Eucommin A		3.2**RRC	1.4*RRC		
494.1213	Salvianolic acid A	2.9*RRC				
578.1999	Podorhizol beta-D-glucoside					1.7*RRC
Flavonoids						
268.0736	7-Hydroxy-4′-methoxyisoflavone		2.5*RRC			
346.0688	5,7,3′,5′-Tetrahydroxy-8,4′-dimethoxyflavone	2.8**RRC				1.6*RRC
366.1467	7-Prenyloxy-3′,4′-dimethoxyisoflavone				1.4*RRC	
410.0308	6-Hydroxykaempferol 7,4′-dimethyl ether 3-sulfate			4.7**RRC		
432.1056	Kaempferol 3-rhamnoside		2.6*RRC	2.4**PRr		
464.1107	Hydnocarpin	1.7*RRC				
506.1060	Quercetin 3-(6″-acetylglucoside)		4.4*RRC	7.7**RRC	4.6**RRC	
506.2305	Exiguaflavanone I	1.6*RRC	1.9*RRC		2.0*RRC	
542.1424	3,4′,5-Trihydroxystilbene 4′-O-beta-D-(6″-O-galloyl)glucopyranoside				1.85**RRC	
578.1636	Kaempferol 7,4′-dirhamnoside	2.3**RRC		3.0*RRC	2.8*RRC	
608.1741	Kaempferol 4′-methyl ether 3-neohesperidoside			1.9*RRC	2.8***RRC	
624.1690	Kaempferide 3,7-diglucoside					3.9*RRC
626.1788	Kuwanon L	2.0*RRC	3.3*RRC	2.5*RRC	2.0*RRC	
638.1483	Kaempferide 5-glucoside-7-glucuronide	3.0***RRC		4.2**RRC	2.4**RRC	5.3*RRC
656.2316	Triphyllin A			8.2**RRC		6.7***RRC
662.1636	Kaempferol 3-(2″,3″-diacetyl-4″-*p*-coumarylrhamnoside	7.6**RRC		5.9**RRC		
674.1999	Nallaflavanone	1.7**RRC		2.1*RRC		
740.2164	Kaempferol 3-isorhamninoside	2.6**RRC		5.1**RRC	2.0**RRC	
754.2320	Rhamnocitrin 3-rhamninoside			2.5*PRr		1.7*PRr
Fatty acids						
210.1256	Jasmonate	3.6**PRr	2.3**PRr	3.0*PRr	3.3**PRr	3.6*PRr
228.1361	Traumatic acid		2.1**RRC		3.0*RRC	
264.2089	Heptadecatrienoic acid	2.3**RRC				
272.2351	2-Hydroxy palmitic acid				1.5*RRC	
278.2246	Linolenic acid	2.9*PRr		3.7*RRC	3.4**PRr	2.8**RRC
280.2402	Linoleic acid	3.8*PRr	1.9**RRC			
282.2559	Oleic acid	1.7**PRr		1.9*RRC		
284.2715	Stearic acid		2.1*PRr		2.8*RRC	

Table 1 (continued)

Accurate Mass	Compound ID	CIho 4196	Zhedar-1	Zhedar-2[a]	Fredrick son	Harbin-2r
288.2300	10,16-dihydroxy-palmitic acid			5.5*RRC		
296.2715	2-Nonadecenoic acid					2.6*RRC
328.2250	9-Hydroperoxy-12,13-epoxy-10-octadecenoic acid			2.8*PRr		

[a] The fold change in abundance in resistant genotype relative to susceptible CH 9520–30; significance level of abundance in resistant relative to susceptible, where *$P<0.05$; **$P<0.01$; ***$P<0.001$; RRC is resistance related constitutive metabolite, PRr is pathogenesis related metabolite in resistant genotype; PRs pathogenesis related metabolite in susceptible genotype. The metabolites that are not significant in particular genotype are left blank.

nin showd the greatest change (3.8×). The TDP was the highest (53 mg kg^{-1}), but the PDC was the second highest (0.74) among genotypes tested (Table 2).

Discussion

Barley genotypes, with different levels of resistance to FHB, varied in their metabolic profiles. We have identified several resistance related constitutive metabolites. These RRC metabolites mainly belonged to three chemical groups: phenylpropanoids, flavonoids, and fatty acids. The occurrence of RRC metabolites, and their magnitude of change in abundance relative to a susceptible genotype, varied across resistant genotypes. The RRC metabolites that occurred in two or more resistant genotypes with at least two fold greater abundance relative to the susceptible were *flavonoids*: several conjugates of kaempferol, quercetin 3-(6″-acetylglucoside), triphyllin A, kuwanon L; *phenylpropanoids*: *p*-coumaric acid, phenylalanine, 4-geranyloxy-5-methyl coumarin, calophyllolide; *fatty acids*: linolenic acid and traumatic acid. Interestingly, several conjugates of kaempherol occurred in high abundance across different genotypes, with the highest increase relative to the susceptible genotype being 7.6× for CIho 4196. All the pathogen inoculated genotypes, not only accumulated DON but also detoxified DON to D3G, in lemma and palea. The most resistant genotype CIho 4196 had the lowest amount of TDP, along with the second highest PDC, the highest being in Zhedar-2 which had moderate TDP. Even though several pathogenesis related metabolites were detected in resistant genotypes, following pathogen inoculation, no resistance-related induced metabolite was identified. Jasmonic acid was a PRr metabolite in all the genotypes, with an up to 3.6× change in abundance, relative to the mock-inoculated. The present study indicates that barley has high levels of constitutive resistance against *F. graminearum*, as has been reported previously in other studies on wheat and barley (Hamzehzarghani et al. 2008; Bollina et al. 2010; Kumaraswamy et al. 2011). These metabolites can be used as potential biomarkers for screening genotypes for resistance against FHB. In addition to metabolites, proteins also significantly contribute to the biochemical resistance mechanisms of the plant against *F. graminearum* (Boddu et al. 2006; Zhou et al. 2006; Golkari et al. 2007; Geddes et al. 2008; Jia et al. 2009).

Metabolic profiling is still an evolving biochemical tool. Several solvents are needed to extract, and different platforms are needed to detect most of the metabolites in plant. Accordingly, we have not detected all the metabolites present in spikelets of the barley genotypes tested here. Using aqueous methanol for extraction and LC-hybrid-MS with a semipolar column (Jupiter) for analysis, we detected more than 1000 metabolites. Still, this column failed to consistently detect DON, 3ADON, and D3G, all of which

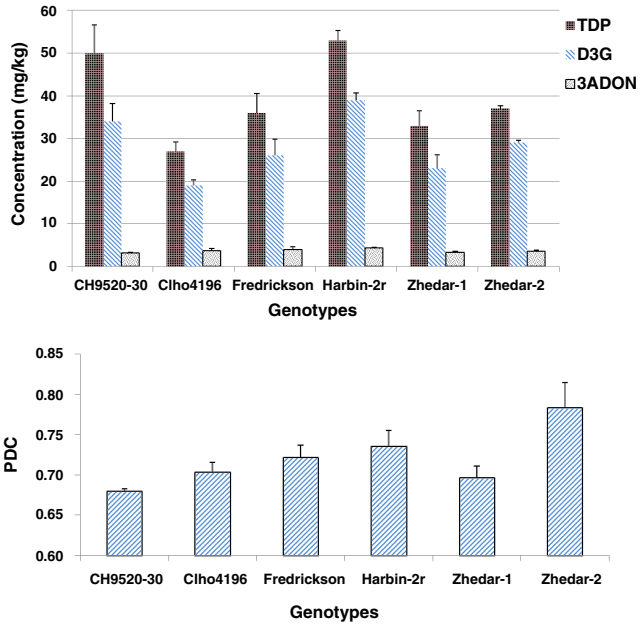


Fig. 3 **a** Total DON produced (TDP = DON + D3G), DON-3-*O*-glucoside (D3G), and 3ADON in lemma + palea of six barley genotypes, following *Fusarium graminearum* inoculation, based on LC-ESI-LTQ-Orbitrap, in mg kg^{-1} of fresh weight of lemma and palea; **b** Proportion of TDP converted to D3G (PDC) in lemma and palea of six barley genotypes

Table 2 Potential biomarker metabolites against *Fusarium* head blight (FHB) in barley: Abundance[a] of metabolites in resistant genotypes relative to susceptible (fold change), total DON produced and proportion of total DON produced converted to DON-3-*O*-glucoside

Biomarkers	CIho 4196	Zhedar-1	Zhedar-2	Fredrickson	Harbin-2r	CH 9520–30
Phenylalanine	**2.1****	2.2*	2.4**	**1.9**	2.0**	
p-Coumaric acid	**2.8***	**1.7**	4.3***	3.1*	**1.6**	
Kaempferol 3-isorhamninoside	2.6**		5.1**	2.0**		
Jasmonic acid	**3.6****	**2.3****	**3.0***	**3.3****	**3.6***	
Linolenic acid	**2.9***	1.4	3.7*	**3.4****	2.8**	
TDP [b]	27***	33**	37**	36**	53	50
PDC[c]	0.70	0.70	0.78**	0.72	0.74	0.68

*$P<0.05$; **$P<0.01$; ***$P<0.001$;

[a] Abundance, expressed in fold change compared to susceptible genotype CH 9520–30; those in normal fonts are RRC and bold are PRr metabolites; blanks = not significant or used as a susceptible control;

[b] TDP = Total DON produced, in mg kg^{-1} of fresh weight of lemma and palea = (DON + D3G);

[c] PDC = Proportion of DON converted = D3G/TDP.

were well-detected with a polar column (Kinetex). Furthermore, we failed to identify any RRI metabolites. This may be due mainly to spray inoculation of spikelets, where the variation in proportion of spikelets infected was high among replicates, which in turn can influence the abundance of metabolites. Individual inoculation of spikelets in wheat has yielded several RRI metabolites, including phenylpropanoids and fatty acids (Hamzehzarghani et al. 2008). In this study, only lemma and palea of the spikelets were analyzed, excluding the rest of the floral organs, as the lemma and palea are the primary defense structures enclosing the ovary/caryopsis, especially in two-row barley that has cleistogamous florets (Yoshida et al. 2007). Although Ma and Bohnert (2007) showed that each organ or tissue type had a specific transcriptome expression in the *Arabidopsis* genome, Golkari et al. (2007) found that the lemma, palea, and glume had similar gene expression patterns, but were different from rachis of wheat inoculated with *F. graminearum*.

Potential Biomarker Metabolites for FHB Resistance

RRC and RI Metabolites The RRC and RI metabolites identified here, which met the following five criteria, were considered as potential biomarkers to screen barley genotypes for resistance against FHB: i) putatively identified (Table S2); ii) high fold change in abundance relative to susceptible; iii) highly significant, especially at $P<0.01$ and $P<0.001$ (Table S1); iv) occurred in more than one genotype; v) associated with plant defense function. Some of these metabolites, or precursors of these metabolites, also were identified as RR metabolites in our previous studies (Bollina et al. 2010; Kumaraswamy et al. 2011). Accordingly, these FHB resistance biomarker metabolites were grouped in their metabolic pathways.

Resistance Indicator Metabolites

Total DON Produced (TDP = DON + D3G) In this study, the most resistant genotype CIho 4196 accumulated the lowest amount of TDP, and moderately resistant genotypes accumulated moderate amounts of TDP, except for Harbin-2r, which accumulated the highest amounts of TDP, similar to the susceptible CH9520-30 (Fig. 3a; Table 2). The accumulation of relatively lower amount of TDP by a resistant genotype can be due to the inhibition of DON/trichothecene synthesis, by the presence of high amounts of antioxidants, especially phenylpropanoids, as has been demonstrated for ferulic acid *in vitro* (Boutigny et al. 2009) and for others (Kushalappa et al. unpublished data). Ferulic acid represses the expression of several trichodiene synthase genes (*Tri-genes*), consequently inhibiting the synthesis of trichothecenes. The amount of DON accumulation in the plant at any time, depends on the amount of DON synthesized, less that converted to D3G. So, the determination of TDP, in this study, has enabled us to better evaluate the factors that affect DON synthesis. DON may not be needed for the initial invasion of spikelets (Jansen et al. 2005), but reduced synthesis of DON may reduce the rate of lesion expansion within florets. However, in our study, the reduced amounts of DON in resistant genotypes also could be due to reduced mycelial biomass relative to susceptible genotypes, even though the DON synthesis can be completely inhibited with little reduction in fungal biomass by phenolic compounds (Boutigny et al. 2009; Kushalappa et al. unpublished data).

Proportion of DON Conversion (PDC = D3G/TDP) Even though RRI metabolites were not detected in our study, a resistance indicator (RI) metabolite, DON-3-*O*-glucoside, an enzymatic detoxification product of DON, by DON-

glucosyltransferase, was detected (Bollina et al. 2010). DON is a virulence factor, and a mutant lacking trichothecene/DON synthesis failed to spread within a spike, in wheat (Proctor et al. 1995). In our study, all six barley genotypes converted DON to D3G, and PDC was the highest by Zhedar-2 (Table 2). Higher conversion ratios of up to 9:1 (D3G/DON) have been reported for wheat recombinant inbred lines with QTL at 3BS, which is associated with resistance to expansion of lesion to spikelets within spike (Lemmens et al. 2005). It is possible that the reduction in TDP and increase in PDC, in lemma and palea of resistant genotypes, as reported here, can lead to reduced expansion of lesion to spikelets through rachis. The ratio of D3G/DON may not correctly identify the mechanisms of resistance, as the DON reduction can be due to both DON conversion to D3G and reduced synthesis (Boutigny et al. 2009).

Phenylpropanoid Pathway

Phenylalanine Phenylalanine, the precursor of the phenylpropanoid and flavonoid pathways, was detected as an RRC metabolite in Zhedar-1, Zhedar-2, and Harbin-2r. Although phenylalanine was not identified as an RR metabolite in our previous studies, several other metabolites related to the phenylalanine ammonia-lyase (PAL) pathway were (Bollina et al. 2010; Kumaraswamy et al. 2011). Although phenylalanine was detected in high amounts in Fredrickson, its level was not significantly different from that in the susceptible genotype. Two transcript-derived fragments TDF83 and TDF17 were strongly up-regulated upon *Fusarium* inoculation in FHB resistant genotypes, and both coded for phenylalanine ammonia-lyase (PAL) (Steiner et al. 2009).

p-Coumaric Acid This was identified as an RRC in Zhedar-2 (4.3×) and Fredrickson (3.1×). It was a PRr metabolite in CIho 4196 (2.8×). *p*-Coumaric acid was identified as an RRC and PRr in our previous studies (Bollina et al. 2010; Kumaraswamy et al. 2011). It inhibited the production of biomass by *F. graminearum* on solid media, with an LD_{50}= 2.5 mM (Kumaraswamy et al. 2011), and an LD_{50}=1.2 mM in liquid media (Bollina et al. 2010). It had a similar effect on both *F. graminearum* and *F. culmorum* at concentrations as low as 0.1 mg mL^{-1} *in vitro* (McKeehen et al. 1999). *p*-Coumaric acid accumulation also was associated with lignification of cell walls (Dixon 2001).

Flavonoid Pathway

Kaempferol Several kaempferol conjugates were identified as RRC metabolites, with up to 7.6× changes in abundance (Table 1). The number of kaempferol conjugates was the highest in Zhedar-2. These also were observed among RRC in our previous studies (Bollina et al. 2010). Naringenin, a precursor of kaempferol, was identified as an RRC metabolite in our previous studies (Bollina et al. 2010; Kumaraswamy et al. 2011). Flavonoids are synthesized in the downstream phenylpropanoid metabolic pathway. The LD_{50} concentration of kaempferol was 4.8 mM on *F. graminearum*, as compared to 1.6 mM for its precursor metabolite naringenin (Bollina et al. 2010). However, the former was not completely soluble in aqueous methanol, thus its conjugates must be tested. Two other flavonoids with high fold change were identified: quercetin 3-(6″-acetylglucoside) (7.7×) and triphyllin A (8.2×), but these metabolites failed to meet all the five criteria used here to select biomarkers.

Fatty Acid Pathway

Jasmonic Acid In this study, JA was identified as a PRr metabolite in all the five resistant genotypes. Even though it was not identified either as a RRC or as a RRI metabolite in this study (PRr not>PRs), it was considered here as a biomarker because of its importance as a signal molecule, especially in necrotrophic pathogenesis. Both JA and methyljasmonate (MeJA) were identified as PRr metabolites in our previous study, and the latter had LD_{50}=1.0 mM for inhibition of mycelial growth on solid media (Kumaraswamy et al. 2011). Plant oxylipins, such as JA and MeJA are synthesized from polyunsaturated fatty acids (Shah 2009). Jasmonic acid and MeJA are the major signaling molecules in establishing resistance against necrotrophic pathogens (Robert-Seilaniantz et al. 2007). In wheat–*F. graminearum* interactions, a total of 14 transcripts were up-regulated, and they were considered to either encode JA biosynthetic enzymes or respond to JA (Jia et al. 2009). Two proteins, ankyrin repeat protein and 12-oxo-phytodienoic acid reductase, related to this signaling pathway were induced 3 days after *F. graminearum* inoculation, in wheat (Zhou et al. 2006). CIho 4196, showed an increased abundance for PR-9 proteins, indicating the possible use of JA antioxidant signaling pathway in pathogen defense (Geddes et al. 2008), which agrees with the results observed in this study.

Linoleic and Linolenic Acids Zhedar-2 and Harbin-2r accumulated linolenic acid as an RRC metabolite. Linolenic acid, however, was also detected as a PRr metabolite in Fredrickson and CIho 4196. Linoleic acid, a precursor of linolenic acid, was identified as an RRC metabolite in Zhedar-1. Both linolenic and linoleic acids inhibited *F. graminearum in vitro* (LD_{50}=3.6 mM for both, based on solid media) (Kumaraswamy et al. 2011). Linolenic acid is

abundant in plasma membranes of wheat cells in the form of phospholipids (Li and Yen 2008), which serve as JA precursors. Linolenic acid can be released by the activities of a membrane-bound phospholipase, and then, oxidized to JA when the cells are under stress.

The potential biomarker metabolites for resistance in barley to *F. graminearum* established here can be used for screening barley genotypes for resistance against FHB, though further elucidation of the occurrence of these in other barley and wheat genotypes can better assess their suitability as biomarkers. Such studies also should identify more biomarkers, as in this study we failed to identify resistance-related induced metabolites, mainly because of high experimental error in spray inoculation. The monoisotopic accurate masses of these biomarkers can be selected in chromatography analyses to speed up the LC-hybrid-MS analysis and output process time. High throughput detection of several metabolites, based on direct infusion (metabolic fingerprinting), can be done in a few minutes. Alternatively, other cheaper tools such as HPLC can be standardized and used. Additionally, comprehensive proteomics can detect the enzymes involved in the biosynthesis of these biomarker metabolites, the sequence of which can be related to genes, which in turn, can be used in DNA marker assisted selections. Metabolic profiling tool, standardized here, can be applied to better understand mechanisms of multiple disease/stress resistance in plants.

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UHPLC-ESI/TOFMS Determination of Salicylate-like Phenolic Gycosides in *Populus tremula* Leaves

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Abstract Associations of salicylate-like phenolic glycosides (PGs) with biological activity have been reported in *Salix* and *Populus* trees, but only for a few compounds, and in relation to a limited number of herbivores. By considering the full diversity of PGs, we may improve our ability to recognize genotypes or chemotype groups and enhance our understanding of their ecological function. Here, we present a fast and efficient general method for salicylate determination in leaves of Eurasian aspen that uses ultra-high performance liquid chromatography-electrospray ionization/time-of-flight mass spectrometry (UHPLC-ESI/TOFMS). The time required for the liquid chromatography separations was 13.5 min per sample, compared to around 60 min per sample for most HPLC protocols. In leaf samples from identical *P. tremula* genotypes with diverse propagation and treatment histories, we identified nine PGs. We found the compound-specific mass chromatograms to be more informative than the UV-visible chromatograms for compound identification and when quantitating samples with large variability in PG content. Signature compounds previously reported for *P. tremoloides* (tremulacin, tremuloidin, salicin, and salicortin) always were present, and five PGs (2'-O-cinnamoyl-salicortin, 2'-O-acetyl-salicortin, 2'-O-acetyl-salicin, acetyl-tremulacin, and salicyloyl-salicin) were detected for the first time in *P. tremula*. By using information about the formic acid adduct that appeared for PGs in the LTQ-Orbitrap MS environment, novel compounds like acetyl-tremulacin could be tentatively identified without the use of standards. The novel PGs were consistently either present in genotypes regardless of propagation and damage treatment or were not detectable. In some genotypes, concentrations of 2'-O-acetyl-salicortin and 2'-O-cinnamoyl-salicortin were similar to levels of biologically active PGs in other Salicaceous trees. Our study suggests that we may expect a wide variation in PG content in aspen populations which is of interest both for studies of interactions with herbivores and for mapping population structure.

Key Words Eurasian aspen · PG · Salicylates · Salicinoids · Genotype recognition · Chemotyping · Diversity · Natural variation

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Introduction

Phenolics are bioactive compounds in plants that play diverse roles including structural support of the cell wall, protection from UV irradiation, and defense from herbivory. These compounds derive from distinct branches of the

shikimate-phenylpropanoid pathway and include the lignins, flavonoids, anthocyanins, salicylate-like phenolic glycosides (PGs), and tannins. The biosynthesis of those phenolic classes that derive from *p*-coumaric acid are particularly well described and include monolignols (Sterky et al., 1998; Boerjan et al., 2003), flavonoids (Tsai et al., 2006; Chen et al., 2009), and condensed tannins (Tanner et al., 2003; Dixon et al., 2005). While PGs are suggested to branch from the shikimate-phenylpropanoid pathway "upstream" of *p*-coumaric acid at either chorismate or cinnamate, their biosynthesis remains poorly understood (Tsai et al., 2006; Morse et al., 2007; Chen et al., 2009; Babst et al., 2010).

Together with condensed tannins, PGs shape resistance against herbivores in the Salicaceous family, including trees of the genera *Populus* and *Salix* (Lindroth and Hwang, 1996; Orians et al., 1997; Albrectsen et al., 2007). In general, anti-insect toxicity appears to increase with the complexity of the PGs, in terms of their molecular weight and number of ring structures (Philippe and Bohlman, 2007).

PG profiles vary qualitatively among species but vary quantitatively within species (Palo, 1984; Julkunen-Tiitto, 1986; Greenaway et al., 1991; Orians et al., 1997). Aspen clones can be recognized by their relative PG contents across environments (Lindroth and Hwang, 1996; Mutikainen et al., 2000; Osier and Lindroth, 2006; Tsai et al., 2006; Donaldson and Lindroth, 2007; Chen et al., 2009). When compared to the entire metabolome, which appears to be both dynamic and diverse (Robinson et al., 2005, 2007), PGs are promising compounds for genotype recognition.

Eurasian aspen (*P. tremula*) is distributed widely across the Northern Hemisphere, and the European part of the population is characterized by a complex genetic structure (De Carvalho et al., 2010). In the Swedish aspen population (SwAsp) of 116 genotypes, this diversity is expressed by phenological variation in bud set and growth differences that mainly follow a latitudinal cline (Luquez et al., 2008). The latitudinal cline also is partly reflected in the distribution of the most abundant and persistent antagonists, rust (*Melampsora sp.*) and a mining moth (*Phyllocnistis labyrinthella*) (Albrectsen et al., 2010). However, the interaction between *P. tremula* and herbivores is poorly understood compared to that of *P. tremuloides* and its antagonists.

The description of PGs for Eurasian aspen has so far been built on information and methods developed for *P. tremuloides*, assuming similar PG profiles (Albrectsen et al., 2010). Advanced methods for chemical profiling increase our ability to explore the diversity and dynamics of defense compounds in plants. The purpose of the present study was to examine the PG profiles of *P. tremula* leaves, using liquid chromatography-mass spectrometry (LC-MS) in order to identify and quantify phenolic glycosides that occur at concentrations indicative of bioactivity. In addition, the study was designed to assess the reliability of this class of phenolic compounds as genotype indicators, and to evaluate their potential as markers to use in future studies of genetic structure and association with resistance traits as assessed through field surveys.

Methods and Materials

Chemicals Salicortin, salicyloyl-salicin, and tremuloidin standards were gifts from S.D. Mansfield (University of British Columbia, Canada), and 2'-O-cinnamoyl salicortin from C. Orians (Tufts University, MA, USA). Tremulacin and salicin were purchased from Sigma (St. Louis, MO, USA). We follow the nomenclature of Zapesochnaya et al. (2002), in which salicyloyl-salicin corresponds to salicoylsalicin according to Kammerer et al. (2005).

Plant Materials and Treatments All plants were *Populus tremula* L. genotypes from the SwAsp Collection (Luquez et al., 2008). The plants had various propagation and treatment histories since one aim of the analyses was to evaluate the robustness of the phenolic glycosides as genotype-specific markers.

For initial characterization and precision analyses, plants representing SwAsp genotypes 7, 13, 47, and 115 were selected. Clones of these genotypes were propagated directly from roots excavated in 2004. The plants were grown in a greenhouse in Umeå, Sweden under ambient conditions since 2005, and cut back so they were about 1 m in height in the fall of 2006. Bud flush occurred in March 2007. In May, when the trees were about 2 m tall, 10 mature leaves were harvested along a 1 m stem section from 2 trees representing each genotype. The harvested leaves were quick-frozen in liquid nitrogen, and stored at −80°C until LC-MS analysis.

Initial Genotypic Profiling For initial assessment of between-genotype differences in phenolic glycoside profiles, we used leaf material from SwAsp genotypes 5, 23, 36, 47, and 72 propagated as described above. We analyzed two biological replicates of each genotype and two technical replicates per plant.

Reliability of PGs for Genotyping To assess the reliability of phenolic glycosides as genotypic markers, we compared their profiles in damaged and undamaged plants. The plants used in this experiment included representatives of SwAsp genotypes 5, 23, 36, 47, 51, and 72, which had been propagated in MS tissue culture medium (Murashige and Skoog, 1962) since May 2007. After transfer to the greenhouse, and a month of acclimatization, on November 9th 2007 (at the height of 55.3±10.8 cm (SD), and with

15.8±3.2 leaves per plant), plants were randomly assigned to three treatments: simulated chewing damage; damage by aphids; and controls.

The aphid used in the experiment was *Chaitophorus populeti* (Panzer). In early September, individuals were collected on Umeå University campus from *Populus tremula*, where they aggregated with two other *Chaitophorus* species, probably *C. pentandrinus* (Ossiannilsson) and *C. vitellinae* (Schrank). Species were determined by Dr. Richa Raizada. Prior to the experiment, cultures of *C. populeti* were kept on aspen plants in the greenhouse where they rapidly developed and reproduced.

In *Populus*, orthostichous leaf positions are 5/10, 6/11 (Jones et al., 1993; Orians and Jones, 2001), i.e., vascular connections are shared between leaf positions 5 and 10, 6 and 11, and so on, with the first fully expanded leaf denoted as 'leaf 1'. The treatments involved punching multiple minute holes into leaves 11–16 (simulated damage treatment), or placing one reproductive female *C. populeti* per leaf (position 11–16, aphid treatment). After 96 h, six mature leaves above the experimental leaves (5–10) were harvested as described above. There were 2–3 biological replicates per genotype and treatment, and one technical replicate per plant (48 plants and samples in total).

Extraction Leaves were ground in a mortar kept frozen using liquid nitrogen, and the frozen powder from each sample (10–12 mg) was extracted by adding it to 1.00 ml of cold chloroform:methanol:H_2O (1:3:1) containing 7 ng/μl of [$^2H_6$]salicylic acid (Isotec, Miamisburg, OH, USA) as an internal standard. The mixture was agitated with a 3 mm tungsten carbide bead in a MM 301 Vibration Mill (Retsch GmbH and Co. KG, Haan, Germany) at 30 Hz for 3 min (Gullberg et al., 2004). To prevent contamination with tissue debris, extracts were centrifuged at 20,800 × *g* for 10 min at 4°C, and 200 μl of the supernatant were evaporated to dryness. The residue was dissolved in 10 μl cold methanol and diluted with 90 μl cold water containing 0.1% formic acid, prior to ultra high performance liquid chromatography (UHPLC)-electrospray ionization/time-of-flight mass spectrometry (ESI/TOFMS). A total sample extraction time of less than 2 h at 4°C, from thawing of leaf tissue to dried extract, was used to control decomposition of phenolic glycosides in the samples. Storage of solutions of the standard compounds at 4°C for this time period did not result in any decomposition of PGs.

UHPLC-TOFMS Analysis The PGs were analyzed by UHPLC-TOFMS using an Acquity™ system, equipped with a column oven (held at 40°C), a 2.1×100 mm, 1.7 μm C18 UPLC™ column and an Acquity photodiode array (PDA) detector coupled to an LCT Premier TOFMS (all from Waters, Milford, MA USA). The PGs in a 2 μl injection of each sample were separated with a mobile phase program consisting of 1–20% B over 0–4 min, 20–40% B (4–6 min), 40–95% B (6–9 min), 95% B (9–13.5 min). Solvent B was acetonitrile containing 0.1% formic acid, solvent A was H_2O containing 0.1% formic acid, and the flow rate was 500 μl min^{-1}. The eluate was passed through the PDA detector for scanning from 210 to 400 nm, at a sampling rate of 20 points s^{-1} and resolution of 1.2 nm, and then directly into the ESI source. The source temperature was 120°C, cone gas flow 10 l h^{-1}, desolvation temperature 320°C, nebulization gas flow 600 lh^{-1}, and the capillary and cone voltages were set at 2.5 kV (negative ionization mode) and 35 V, respectively. Data were acquired, in dynamic range enhancement (DRE) mode, every 0.1 s, with a 0.01 s interscan delay. Leucine enkephalin was the lock mass compound for accurate mass measurements, infused directly at 400 pg μl^{-1} in 50:50 acetonitrile:H_2O at 20 μl min^{-1}. The normal lock mass in DRE mode was the negative ^{13}C ion of leucine enkephalin (*m/z* 555.2654), and the extended lock mass was the normal negative ion at *m/z* 554.2620. Mass spectra were acquired in centroid mode, *m/z* 100–1000, with a data threshold value set to 3. In a complementary experiment, in-source fragmentation was increased by increasing the aperture 1 voltage from 5 to 45 V to exclude adduct ion formation.

Profiling–Data Processing The UHPLC-ESI/TOFMS instruments were operated with MassLynx™ v. 4.1 software (Waters, Milford, MA, USA), and the QuanLynx module of this package was used to assess relative amounts of phenolic glycosides by calculating peak areas from both mass chromatograms (see Table 1, ESI-MS *m/z* with 0.10 Da mass windows), and specific wavelength chromatograms using the compound-specific UV λ_{max} values (Table 1). The peak areas were normalized against that of the internal standard salicylic acid (M = [$C_7H_2{}^2H_4O_3$], RT = 5.01 min, *m/z* 141.0495, 187.0550, and 305.0883 for the $[M\text{-}H]^-$, $[M\text{-}H + HCOOH]^-$ and $[M_2{-}2H + Na]^-$ ions, respectively; λ_{max} 231 and 305 nm), as well as the mass of tissue used for extraction.

Phenolic Glycoside Structural Identification (UHPLC-LTQ/Orbitrap) To elucidate the structures of the compounds, they were separated by a Thermo Accela LC system, equipped with a column oven (held at 40°C) and a Hypersil C18 GOLD™ column (2.1×50 mm, 1.9 μm; mobile phase as for the UHPLC-ESI/TOFMS) and analyzed by tandem mass spectrometry using a LTQ/Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Centroid mass spectra of negative ions were collected in the Orbitrap mass analyzer, with a target mass resolution of 30,000 (full width at half maximum peak height, defined at *m/z* 400), after collision-induced dissociation (CID) in the LTQ cell,

Table 1 Salicylate-like phenolic glycosides in *Populus tremula* leaf extract identified by retention time (RT), electrospray ionization mass spectrometry (ESI-MS) and photodiode array detector (UV)

Name	Molecular formula	RT (min)	Theoretical *m/z* $(M-H)^-$; $(M-H+HCOOH)^-$	ESI-MS	[m/z]	UV λmax[a] (nm)
Salicin	$C_{13}H_{18}O_7$	2.37	285.0980; 331.1035	331.110[b](+20 ppm)	[285.12] [c]	212, 267[d]
2'-O-acetyl-salicin	$C_{15}H_{20}O_8$	3.73	327.1085; 373.1140	373.123 (+24 ppm)	[327.11]	212, 269
Salicortin	$C_{20}H_{24}O_{10}$	4.55	423.1297; 469.1351	469.175 (+85 ppm)	[423.13]	216, 270[d]
2'-O-acetyl-salicortin	$C_{22}H_{26}O_{11}$	5.45	465.1402; 511.1457	511.185 (+77 ppm)	[465.14]	213, 269
Tremuloidin	$C_{20}H_{22}O_8$	5.72	389.1242; 435.1297	435.169 (+90 ppm)	[389.13]	219, 270[d]
Salicyloyl-salicin	$C_{20}H_{22}O_9$	6.37	405.1191; 451.1246	405.164(+101) + 451.170(+111)		240, 270, 304[d]
Tremulacin	$C_{27}H_{28}O_{11}$	6.48	527.1559; 573.1614	573.199 (+66 ppm)	[527.16]	220, 270[d]
2'-O-cinnamoyl-salicortin	$C_{29}H_{30}O_{11}$	6.82 & 6.95	553.1715; 599.1770	599.213 (+60 ppm)	[553.17]	218, 278[d]
Acetyl-tremulacin	$C_{29}H_{30}O_{12}$	7.04	569.1665; 615.1719	615.182 (+16 ppm)	[569.18]	221, 272

a = λmax in UV spectrum from the PDA detector

b = compound mass detected in MS spectrum of plant extract (aperture 1 = 5 V), average *m/z* from 10 samples; with mass accuracy expressed in ppm (in parenthesis)

c = additional *m/z* detected with increased in-source dissociation (aperture 1 = 45 V), average *m/z* from 3 samples

d = data from standard compound

using a normalized collision energy of 20%. External mass calibration was performed according to the manufacturer's guidelines.

Analytical Precision To assess the precision of the analyses, 5 samples of leaves of each of 2 genotypes (7 and 115) were extracted, and part of each extract was analyzed in parallel, while the other parts were pooled by genotype. To test for methodical errors, the pooled extracts were each divided into 10 microvials, and analyzed by UHPLC-PDA-ESI/TOFMS. In addition, 5 repeat injections from 1 individual microvial were performed to determine injection and instrumentation errors. To check for retention time and mass accuracy throughout the experiment, a standard mix consisting of 5 standards (salicin, chlorogenic acid, rutin, nalidixic acid, and phloretin), each at 50 μM in 50% acetonitrile, was injected after every ten injections.

Concentration Determination Standards of various concentrations (0.5 ng–10 μg injected) were analyzed by UHPLC-PDA-ESI/TOFMS as described above, and the peak areas from the resulting mass chromatograms were used to construct calibration curves.

Statistical Analysis Multivariate analysis of PG profiles was performed using principal component analysis (PCA), with unit variance scaling, in SIMCA-P+ v. 12.0 (Umetrics AB, Umeå, Sweden). Relative standard deviations (RSD) were measured for reproducibility and RSD in the Umetrics software equals the coefficient of variation (CV).

Results

Identification With LC-ESI/TOFMS operating in negative ionization mode, we identified nine salicylate-like phenolic glycosides in *P. tremula* leaves: salicin, salicortin, tremuloidin, tremulacin, 2'-O-acetyl-salicin, 2'-O-acetyl-salicortin, salicyloyl-salicin, 2'-O-cinnamoyl-salicortin, and acetyl-tremulacin (Fig. 1, Table 1). The identifications were based on comparisons of the LC-retention times, UV spectra, and accurate masses of the compounds detected in the extracts with those of standards (Table 1). These nine phenolic glycosides were baseline-separated by the LC (Fig. 2a), and they constituted some of the most intense peaks in the total ion chromatograms, suggesting that they occurred in high concentrations (Fig. 2b).

Phenolic glycosides are aromatic, and thus can be detected by monitoring UV absorbance. The summed PDA absorbance (UV) chromatograms (Fig. 2c) had fewer peaks than the total ion chromatograms acquired by TOFMS (Fig. 2b). However, the peaks that were detected displayed characteristic absorbance spectra of phenolic glycosides, with two UV peaks (Fig. 2c insert and Table 1), one at about 215–220 nm (but 240 nm for salicyloyl-salicin), and a weaker one at 270 nm. Either wavelength could be used for quantification (Table 2).

A drawback of using ESI-MS is the formation of adducts. With the ESI/TOFMS instrument used in these analysis, adducts with acetonitrile, formic acid and/or sodium ion have been detected in the negative ionization mode, and ammonium adducts found in the positive mode. For all of the phenolic glycosides, the mass spectra showed a base peak of the deprotonated molecular ion as a formic

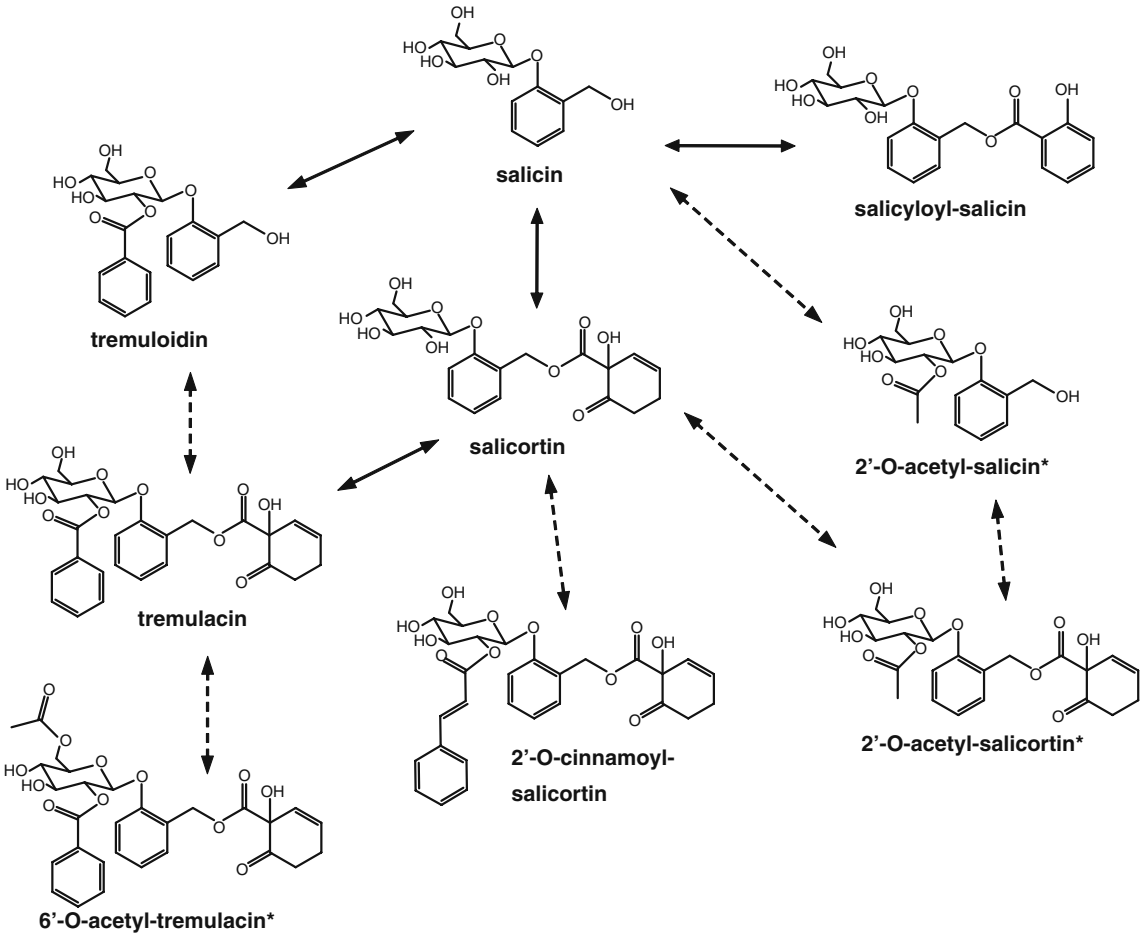
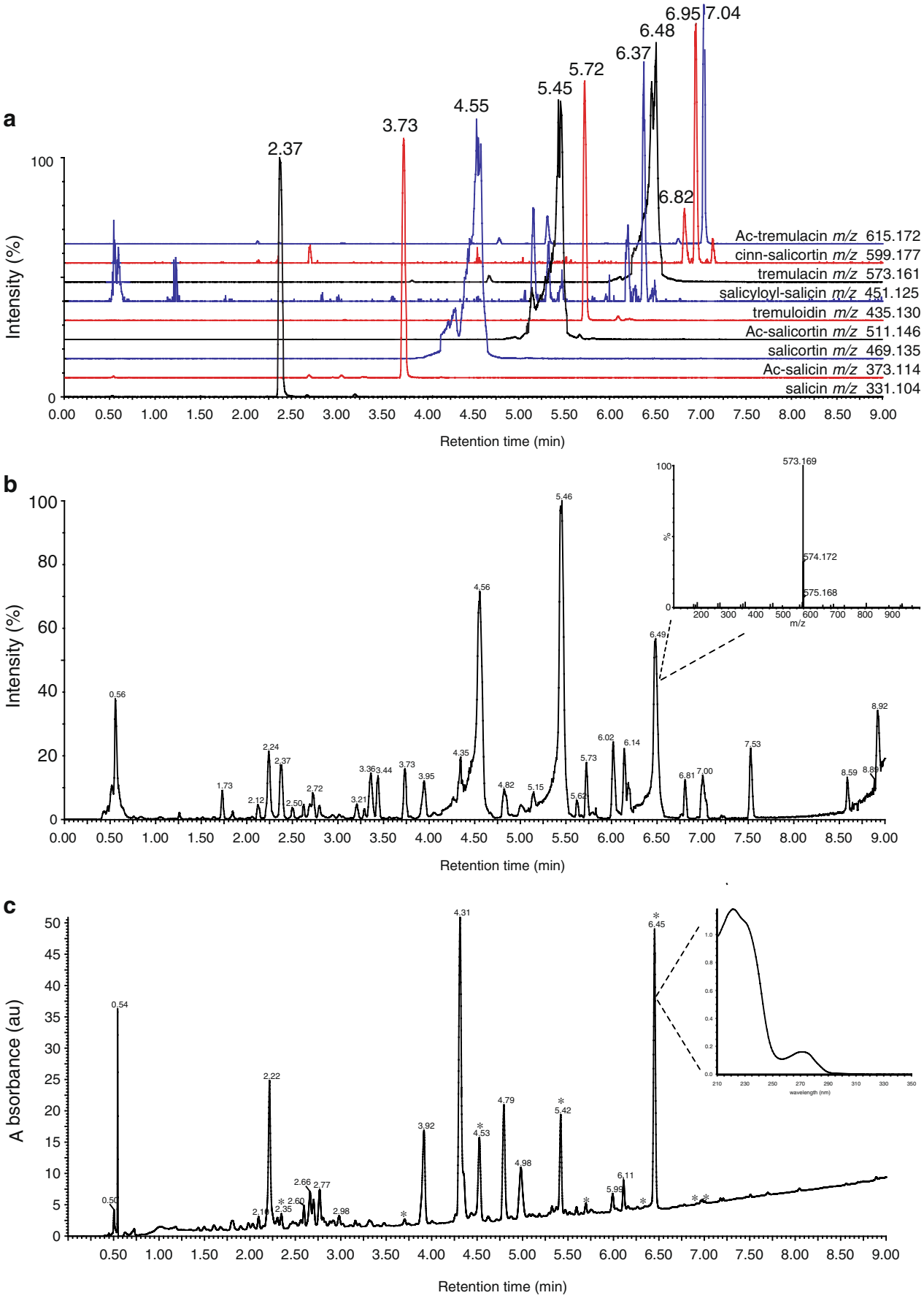


Fig. 1 Nine phenolic glycosides identified in leaf tissue of *Populus tremula*. The structures marked with *asterisks* are tentative. *Solid lines* indicate suggested biosynthesis pathways from the literature, and *dashed lines* pathways inferred from structural similarities

acid adduct $[M\text{-}H + HCOOH]^-$, presumably arising from combination with the formic acid present in the LC mobile phase. The intensity of the unmodified molecular ion was very low (Fig. 2b insert and Table 1) for the PGs, except for salicyloyl-salicin in the samples as well as in pure standards. In the complementary analysis with increased in-source dissociation in the ESI/TOFMS, the deprotonated molecular ion and the formic acid adduct were both present, confirming the phenolic glycoside masses.

The initial identifications of the three novel acetylated phenolic glycosides were based on their masses and absorbance spectra. To verify their identities, the extracts also were analyzed by LC-MS/MS using an LTQ/Orbitrap system (Hu et al., 2005; Makarov et al., 2006) (Fig. 3, Suppl. Fig. S1) and structural identification of the novel phenolic glycosides was obtained from the fragmentation patterns. The ESI system of this instrument also generated formic acid adducts, but it yielded deprotonated, unmodified ions at higher intensities, and the latter were preferentially selected by mass for the MS/MS analysis. Utilizing the high mass accuracy of the Orbitrap MS, the elemental compositions of these phenolic glycosides confirmed that they consisted of acetylated versions of salicin, salicortin, and tremulacin. The acetyl-salicortin (Fig. 3b) fragments were similar to those from salicortin (Suppl. Fig. S1d; Kammerer et al., 2005), with several fragments that increased by 42 Da in the acetyl-salicortin spectrum. Therefore, we concluded that the acetyl group was attached to the glucose moiety of the salicortin. Similar conclusions were drawn for acetyl-salicin (Fig. 3a) and acetyl-tremulacin (Fig. 3e). The characteristics of the UV spectra, as well as the slightly longer retention times compared to those of the non-acetylated phenolic glycosides (Table 1), corroborated our structural assignment. We suggest that the acetyl group of acetyl-tremulacin is bonded to the 6'-O of the glucose ring, since this seems to be the second most reactive position, as in diacetyl-salicortin (Ruuhola et al., 2003; Ruuhola and Julkunen-Tiitto, 2003). We propose that the 2'-O position is occupied by the benzoyl moiety in tremulacin, and that the acetyl groups of acetyl-salicin and acetyl-salicortin occupy the 2'-O-positions of salicin and salicortin, as found in willows (Ruuhola and Julkunen-Tiitto, 2000).


a
Intensity (%)
Retention time (min)
Ac-tremulacin m/z 615.172
cinn-salicortin m/z 599.177
tremulacin m/z 573.161
salicyloyl-salicin m/z 451.125
tremuloidin m/z 435.130
Ac-salicortin m/z 511.146
salicortin m/z 469.135
Ac-salicin m/z 373.114
salicin m/z 331.104
b
Intensity (%)
Retention time (min)
573.169
574.172
575.168
m/z
c
A bsorbance (au)
Retention time (min)
wavelength (nm)

◀ **Fig. 2** UHPLC-PDA-ESI/TOFMS analysis of a plant extract (genotype 47). **a** Single mass chromatograms (*m/z* window 0.04) for the nine phenolic glycosides. **b** Total ion chromatogram showing relative intensities of peaks from TOFMS, with the mass spectrum for tremulacin inserted. **c** Full wavelength chromatogram from the PDA detector (UV, 210–400 nm) with the absorbance spectrum for tremulacin (peak at 6.45 min) inserted. Since the PDA detector was connected between the LC-column and the TOFMS, the retention time was *ca.* 0.3 min shorter than in MS detection. *Asterisks* indicate peaks corresponding to phenolic glycosides. The LC-retention times are indicated

Method Reproducibility To validate the UHPLC-PDA-ESI/TOFMS method for analyzing phenolic glycosides we used it to analyze multiple sub-samples from the same (pooled) extract. The reproducibility was within acceptable ranges for detectable peaks with RSDs, of 4–7% for UV detection, and 6–12% for MS detection (Table 2). Sequential injections from the same microvial showed little variation, with RSDs as low as <2% for UV, and 1–5% for MS quantification (Table 2), indicating little instrumental variation. Parallel extractions from subsamples of the same plant tissue were performed to explore the reproducibility of all steps in the analysis, and resulted in somewhat higher variations (Table 2).

Phenolic Glycoside Concentrations Six standards (salicin, salicortin, tremuloidin, salicyloyl-salicin, tremulacin, and 2'-O-cinnamoyl-salicortin) at various concentrations were analyzed by UHPLC-PDA-ESI/TOFMS, and the calibration curves obtained from the resulting mass chromatograms were used for quantification. The UV and MS detection limits for individual phenolic glycosides varied (Table 3), but mass spectrometry could generally detect lower concentrations. Therefore, UV detection may not be optimal when screening several genotypes with highly variable phenolic glycoside concentrations. In such cases, MS detection may be more suitable for quantification, at the expense of somewhat lower reproducibility (Table 2).

Phenolic glycosides concentrations were calculated per unit mass of leaf powder (Table 3). Salicin was found at 0.5–2.4 μg mg^{-1} and tremulacin at 0.1–4.0 μg mg^{-1}, depending on the genotype. These levels are comparable to concentrations found in *P. tremuloides* (Lindroth and Hwang, 1996; Robinson et al., 2005; Harding et al., 2009). In some genotypes, 2'-O-cinnamoyl-salicortin was detected in concentrations up to 2.1 μg mg^{-1}, which is within the range of 0.75–5.6 μg mg^{-1} found in *Salix sericea* (Orians and Fritz, 1995). In other genotypes, this compound was below the detection limit of about 0.25 μg/mg tissue. Salicortin, tremuloidin, and salicyloyl-salicin were found within ranges of 1.8–6.9, 0.1–1.1, and 0–0.12 μg mg^{-1}, respectively. Preliminary calculations based on the assumption that concentration/signal intensity ratios

Table 2 Reproducibility of the UHPLC-PDA-ESI/TOFMS analysis method. Plant extracts (of *Swasp* genotype 115) were analyzed, peak areas in mass/wavelength chromatograms[1] were extracted (window of 0.10 Da resp 0.15 nm), normalized[2] and relative standard deviation (RSD)[3] were calculated

Compound	Multiple injections[a]			Multiple microvials[b]			Multiple extractions[c]		
	~215 nm	~270 nm	*m/z* (M-H + HCOOH)$^-$	~215 nm	~270 nm	*m/z* (M-H + HCOOH)$^-$	~215 nm	~270 nm	*m/z* (M-H + HCOOH)$^-$
Salicin	1.6%	1.1%	4.8%	5.9%	5.9%	11.7%	8.3%	9.2%	16.9%
2'-O-acetyl-salicin	1.0%	0.6%	4.4%	5.7%	5.6%	10.6%	14.0%	14.1%	13.7%
Salicortin	1.4%	0.9%	3.6%	5.8%	5.4%	10.2%	7.0%	7.6%	27.5%
2'-O-acetyl-salicortin	0.8%	0.8%	2.9%	5.0%	5.8%	10.5%	11.5%	11.7%	20.1%
Tremuloidin	0.7%	1.0%	2.4%	6.0%	5.8%	10.6%	15.9%	15.4%	16.5%
Salicyloyl-salicin	N/D	N/D	6.3%[d]	N/D	N/D	6.3%[d]	N/D	N/D	26.2% [d]
Tremulacin	0.9%	1.0%	2.5%	5.5%	5.1%	10.8%	12.7%	13.4%	18.4%
2'-O-cinnamoyl-salicortin	N/D	7.1%	15.5%	N/D	6.0%	6.0%	N/D	22.0%	29.6%
Acetyl-tremulacin	6.7%	2.7%	8.7%	6.5%	7.0%	10.3%	19.5%	12.5%	20.9%

1 = for compound specific m/z and UV λmax see Table 1

2 = against internal standard (and weight of tissue in the case of multiple extractions)

3 = same as coefficient of variation (CV)

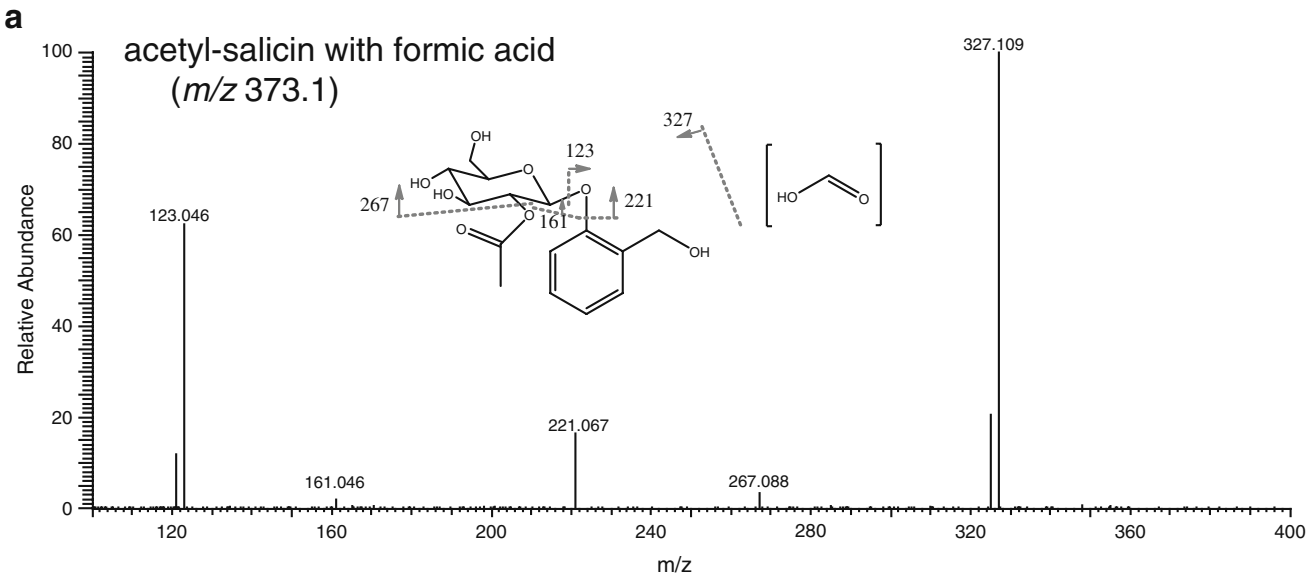
a = five repetitive injections from the same microvial

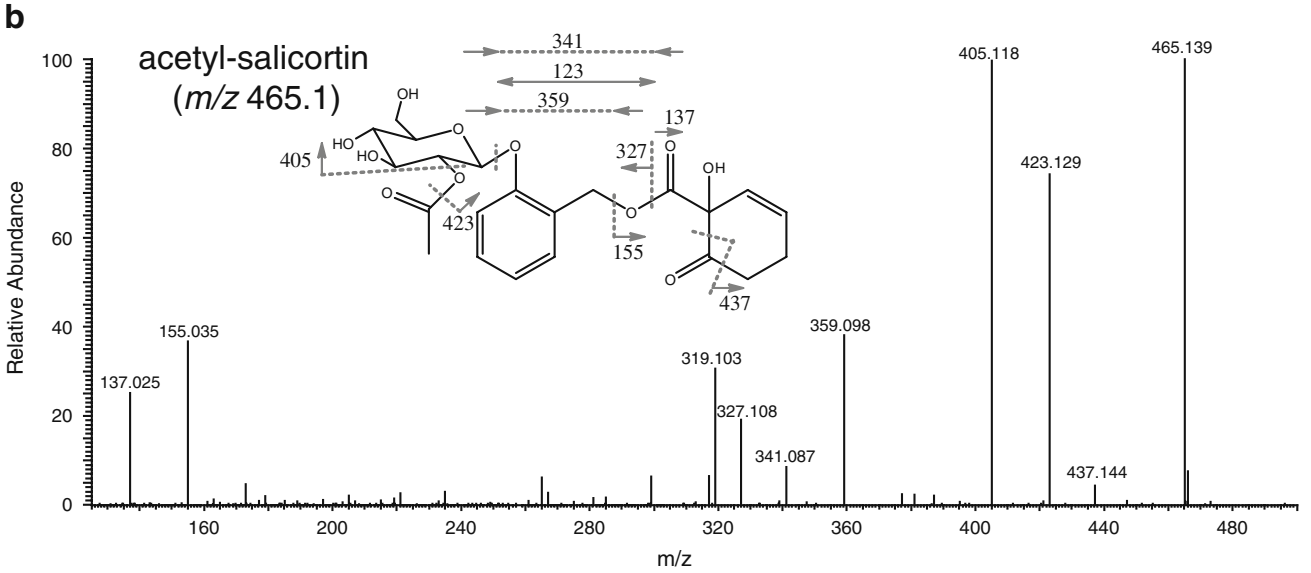
b = ten microvials with same plant extract

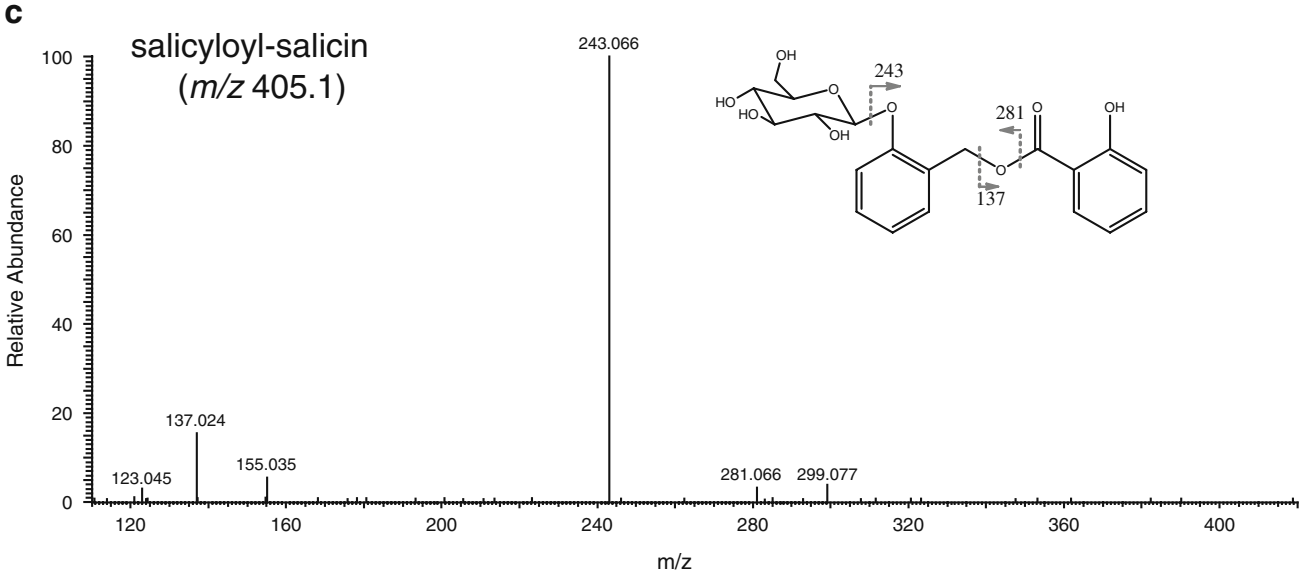
c = five parallel plant extractions analyzed

d = sum of peak area in mass chromatograms of $(M-H)^-$ and $(M-H + HCOOH)^-$

N/D = not detectable peak


a
acetyl-salicin with formic acid
(m/z 373.1)
Relative Abundance
m/z
327.109
123.046
221.067
161.046
267.088
123
221
267
161
327


b
acetyl-salicortin
(m/z 465.1)
Relative Abundance
m/z
405.118
465.139
423.129
155.035
137.025
359.098
319.103
327.108
341.087
437.144
341
123
359
137
405
327
423
155
437


c
salicyloyl-salicin
(m/z 405.1)
Relative Abundance
m/z
243.066
137.024
123.045
155.035
281.066
299.077
243
281
137

◀ **Fig. 3** CID MS/MS spectra of the novel phenolic glycosides: **a** 2'-O-acetyl-salicin, **b** 2'-O-acetyl-salicortin, **c** salicyloyl-salicin, **d** 2'-cinnamoyl-salicortin, and **e** acetyl-tremulacin. Respective *m/z* selected for fragmentation is indicated. Compound structures are shown with proposed bond breakages during fragmentation

were similar to the corresponding unacetylated compounds suggest that acetyl-salicin, acetyl-salicortin, and acetyl-tremulacin were present at concentrations ranging from 0 to 1.9, 0 to 2.9, and 0 to 0.2 μg mg^{-1}, respectively.

Phenolic Glycosides as Genotype-Specific Markers PCA of the data acquired in the initial assessment of between-genotype differences in phenolic glycoside profiles suggests that the five tested genotypes clustered into three distinct chemotype-groups (score plot, Fig. 4a and Fig. S3) and that the nine phenolic glycosides all contributed to this pattern (loadings, Fig. 4a). The PCA model showed two significant components, explaining 81% of the variation in the data [R^2X(cum) = 0.812; Q^2(cum) = 0.565]. Variations in contents of 2'-O-cinnamoyl-salicortin, the acetylated phenolic glycosides, and to some extent tremulacin and tremuloidin were largely responsible for separating the genotypes along the first principal component axis (PC1).

Analysis of the phenolic glycoside profiles of representatives of an overlapping set of genotypes, following varying propagation and stress treatments, suggested a similar pattern; six genotypes formed two distinct clusters or chemotype-groups (Fig. 4b-I). One chemotype-group included three separate genotypes (47, 51, and 72) that contained acetylated phenolic glycosides, while the other included three genotypes (5, 23, 36) characterized by the presence of 2'-O-cinnamoyl-salicortin (Fig. 4b-I). Again, all

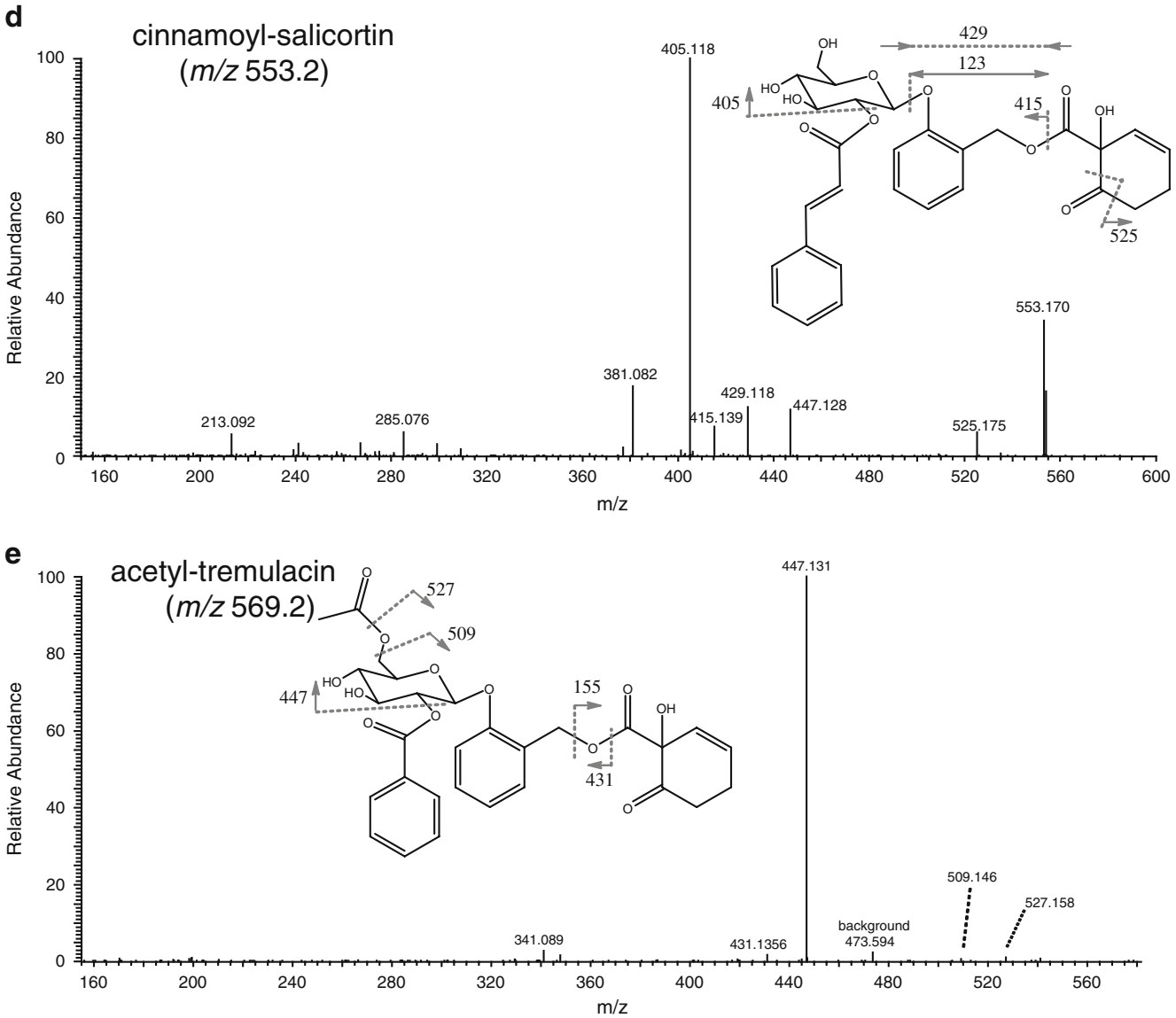


Fig. 3 (continued)

Table 3 Concentrations of salicylate-like phenolic glycosides in *P. tremula*

Compound	Lower detection limits[a]				Highest detected
	~215 nm	~270 nm	*m/z* $(M-H + HCOOH)^-$	(μg salicylate/mg tissue)[b]	conc[c] (in genotype)
Salicin	2 ng	2 ng	1 ng	0.03 μg/mg	2.4 μg/mg (gen72)
2'-O-acetyl-salicin	N/A	N/A	N/A	N/A	1.9 μg/mg (gen47) [d]
Salicortin	10 ng	2 ng	4 ng	0.10 μg/mg	6.9 μg/mg (gen72)
2'-O-acetyl-salicortin	N/A	N/A	N/A	N/A	2.9 μg/mg (gen47) [d]
Tremuloidin	2 ng	5 ng	2 ng	0.05 μg/mg	1.1 μg/mg (gen47)
Salicyloyl-salicin	30 ng	30 ng	5 ng[e]	0.12 μg/mg	<0.12 μg/mg
Tremulacin	20 ng	30 ng	5 ng	0.12 μg/mg	4.0 μg/mg (gen72)
2'-O-cinnamoyl-salicortin	50 ng	50 ng	10 ng	0.25 μg/mg	2.1 μg/mg (gen5)
Acetyl-tremulacin	N/A	N/A	N/A	N/A	0.2 μg/mg (gen51) [d]

a = Several concentrations of standards were injected and peak areas in mass/wavelength chromatograms extracted (window of 0.10 Da resp 0.15 nm). For compound specific m/z and UV λmax see Table 1

b = lower detection limits recalculated per unit weight of leaf tissue using mass chromatograms values

c = recalculated using mass chromatograms in extracts from the two datasets

d = constant for non-acetylated compounds used for calculations in tissue

e = sum of peak area in mass chromatograms of $(M-H)^-$ and $(M-H + HCOOH)^-$

N/A = standard not available

phenolic glycosides contributed to the clustering of the genotypes (loadings, Fig. 4b), although 2'-O-cinnamoyl-salicortin had the heaviest loading on the first axis (PC1). Since PCA did not detect any treatment effects (Fig. 4b-II), the phenolic glycosides appear to be potentially robust chemotype group markers and, to some extent genotype markers in *P. tremula*. The PCA model constructed from the data acquired from the analyses of samples of plants subjected to various treatments had four significant principal components, which together explained 96% of the variation in the data [R^2X(cum) = 0.955; Q^2(cum) = 0.831].

The consistent separation into chemotype-groups in the two data sets (Fig. 4a and b) was confirmed by studies of separate profiles for each plant and phenolic glycoside compound (Suppl. Fig. S2, showing normalized data obtained in the PG consistency analysis). Depending on the genotype, the phenolic glycosides 2'-O-acetyl-salicin, 2'-O-acetyl-salicortin, 2'-O-cinnamoyl-salicortin, and acetyl-tremulacin, were either consistently present in substantial amounts, or consistently below the detection limit. The structural similarities of the compounds that contributed significantly to the patterns detected in the multivariate analyses are indicated in the loading plot (Suppl. Fig. S3).

Discussion

Nine phenolic glycosides were found by UHPLC-ESI/TOFMS in extracts of European aspen (*P. tremula*) leaves. Salicin, salicortin, tremuloidin, and tremulacin were present in all tested genotypes, in agreement with the "signature" phenolic glycosides reported for *P. tremuloides* (Lindroth and Hwang, 1996; Harding et al., 2009). Salicyloyl-salicin also was found in most extracts. In addition, some genotypes contained considerable amounts of 2'-O-acetyl-salicin, 2'-O-acetyl-salicortin, 2'-O-cinnamoyl-salicortin, and/or acetyl-tremulacin, which have not been described previously in either *P. tremula* or *P. tremuloides*.

Strengths of UHPLC-MS A TOFMS instrument is suitable for identifying phenolics, when standard compounds are available, but it has limited utility for identifying novel compounds, since structural elucidations require MS/MS analysis for identification and confirmation. For this purpose, the high mass-accuracy LTQ/Orbitrap instrument was used here, although other MS/MS compatible instruments could have been used with equally good results. Selected reaction monitoring (SRM) methods using an instrument such as triple quadrupole (QQQ) might also have been used for quantification purposes, but would require prior knowledge about compound masses. Well established HPLC protocols take 40 to 60 min per sample to analyze phenolic glycosides (Ruuhola et al., 2003; Ruuhola and Julkunen-Tiitto, 2003; Warren et al., 2003). However, by using ultra high performance liquid chromatography (UHPLC), the analyses are faster and the resolution is better, resulting in higher precision. The methodology for phenolic glycosides presented here reduced the time required for the liquid chromatography separations to just 13.5 min.

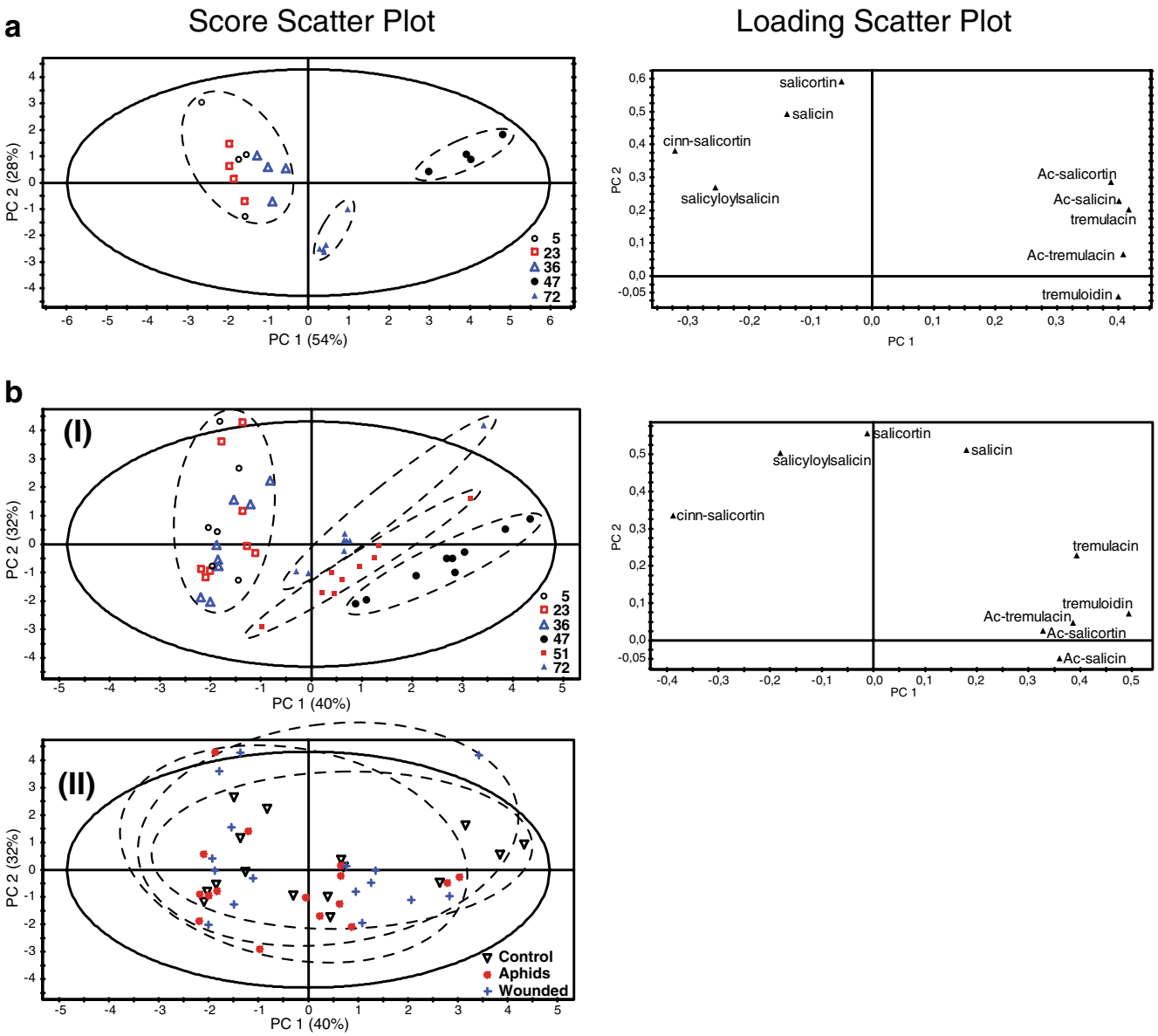


Fig. 4 Multivariate analysis of phenolic glycoside profiles of *Populus tremula* genotypes from the SwAsp collection. **a** PCA score plot (PC1 versus PC2) of data acquired from analyses of samples of greenhouse-grown plants representing five genotypes (5, 23, 36, 47, and 72), with data marked by genotype, and the corresponding loading plot. **b** Corresponding plots of data acquired from analyses of plants representing six genotypes (5, 23, 36, 47, 51, and 72) exposed to three treatments: simulated damage (= wounded), aphid infestation and controls, with data coded by (I) genotype, and (II) treatment. *Solid circles* show 95% Hotelling T^2 confidence regions, and *dashed circles* indicate chemotype-groups (in a, b-I), and treatment groups (in b-II)

Our results also showed the limitation of detection by UV, in comparison to MS. Although UV detection generally provided the best reproducibility for individual phenolic glycosides, it is less sensitive than MS for phenolic glycosides. When large numbers of samples with variable concentrations are to be analyzed, MS measurements are more suitable, since they can cope with a wider range of concentrations. In addition, an extracted mass chromatogram is more compound specific than an extracted UV-visible chromatogram; in the latter, contributions from coeluting compounds are harder to distinguish and might affect the quantification. Hence, the TOF and Orbitrap MS analysis was essential for characterizing the phenolic glycosides in the aspen leaf extracts. Depending on the objective, less powerful MS systems might be sufficient, although a rapidly scanning instrument is highly advantageous for collecting many data points over each chromatographic elution peak. While in recent years hundreds of UHPLC-base methods have been published for analyzing various classes of metabolites, this is the first method for phenolic glycoside analysis to date.

Can Phenolic Glycoside Profiles Be Used to Characterise Populus-Genotypes? Multivariate (PCA) analysis of the PG profiles clustered the genotypes into similar groups, regardless of the treatments applied (Fig. 4). Hence, the phenolic glycosides provided a means for recognizing chemotype groups of *P. tremula* across treatments. Salicin, salicortin, tremuloidin, and tremulacin were present in all samples and varied with genotype. These four compounds are well-known to vary quantitatively with *P. tremuloides* genotype (Lindroth and Hwang, 1996; Harding et al., 2009). In contrast, the five novel phenolic glycosides in *P. tremula* followed a discrete pattern of presence or absence in a given genotype. Moreover, when present, the novel compounds also varied in quantity in a genotypic specific pattern, and contents of 2'-O-cinnamoyl-salicortin, and the acetylated phenolic glycosides were largely responsible for separating the genotypes along the first principal component axis. (for profiles of particular phenolic glycosides see progeny data provided in Suppl. Fig. S2). The pattern of appearance of these novel phenolic glycosides suggests that they are potential Mendelian traits i.e., traits controlled by a single loci. Such traits are promising for studies of both herbivore resistance and genetic structure. Populations of *P. tremula* are polymorphic, and thus genetically highly variable for several growth traits (Ingvarsson, 2005; De Carvalho et al., 2010), many of which do not follow simple Mendelian inheritance (Luquez et al., 2008). The novel phenolic glycosides not only confirm that aspens in Sweden may constitute a complex genetic composition, but also offer new traits that may be helpful in understanding the population structure of Eurasian aspen.

Can We Expect to Find Other PGs in P. tremula? Novel phenolic glycosides were detected by identifying compounds with salicylate-like UV-spectra combined with LC-ESI/TOFMS-analysis. Peaks that had similarities to known phenolic glycosides were analyzed further by LTQ-Orbitrap MS, resulting in accurate mass determination, and tandem mass spectra for structural interpretation. At the population level, there may be more salicylate-like phenolic compounds to discover in *P. tremula*, and only careful survey of a larger number of genotypes will provide a comprehensive list of compounds. Many salicylate-like phenolic glycosides have been described in Salicaceous species (Ruuhola and Julkunen-Tiitto, 2000, 2003; Kammerer et al., 2005; Tsai et al., 2006), and although we can exclude the possibility that additional PGs were present in the major peaks in the samples that we screened, more genotypes should be screened to obtain information about their diversity at stand and population levels. In the samples examined in this study, the phenolic glycosides always were associated with formic acid under our LC-MS conditions, which led to the detection of acetyl-tremulacin. Thus, assuming that an acetylated tremuloidin compound would also associate with formic acid, we specifically searched for such a compound (using *m/z* 431.1348 and 477.1402 for the compound with and without a formic acid adduct) and concluded it was not present in our samples in detectable amounts.

Implications of PG Diversity Biosynthetic routes of the salicylate-like phenolic glycosides have proven less easy to elucidate compared to those for flavonoids (Tsai et al., 2006; Chen et al., 2009; Babst et al., 2010). On the basis of correlated patterns in the loading plots (Fig. 4), the biosynthesis of acetylated phenolic glycosides appear related to that of non-acetylated molecules with similar structures (Fig. 1), thus supporting conclusions drawn by Ruuhola and Julkunen-Tiitto (2003). Moreover, some phenolic glycosides might be metabolically interconverted (Ruuhola and Julkunen-Tiitto, 2003; Babst et al., 2010), a possibility which would agree with the close correlation between salicin and salicortin in our loading plots. Salicin and salicortin appear on the second principal component axis in the PCA plots, rather than the first component, which is responsible for the main separation among genotypes, providing further support for a dynamic interrelationship between salicin and salicortin.

Phenolic glycosides have well-established biological activities and utility for distinguishing among genotypes within and among species. Salicortin and tremulacin, for example, are associated with resistance to defoliating insect herbivores in *P. tremuloides* (Hemming and Lindroth, 1995; Osier and Lindroth, 2006; Donaldson and Lindroth, 2007; Wimp et al., 2007), while salicortin and 2'-O-cinnamoyl-salicortin are associated with resistance to a range of herbivores within the North American *Salix sericea* × *eriocephala* hybrid complex (Orians et al., 1997). Diverse phenolic glycoside composition also has been identified in Finnish and Swedish *Salix* spp., including 2'-O-acetyl-salicin and 2'-O-acetyl-salicortin in *S. pentandra* (Ruuhola and Julkunen-Tiitto, 2003); although in these species, the relationships between specific compounds and biological activities appear to be complex or non-existent, thus highlighting the ambiguous ecological functions of phenolic glycosides (Hjältén et al., 2007; Witzell and Martin, 2008; Albrectsen et al., 2010).

Compared to other Salicaceous study systems, we have little information regarding biological activity of phenolic glycosides in *P. tremula*. Generally, the intra-specific diversity expressed in the modest number of genotypes in the present study suggests that the phenolic glycoside composition of *P. tremula* is more complex than that of *P. tremuloides*. Given the great differences in damage regimes in the two aspen species and the defensive role that phenolic glycosides often play against various pests, the

differences in distribution of defense compounds, warrant further attention. Hence, a detailed comparative survey of phenolic glycoside diversity at population level in North American and Eurasian aspen may help to elucidate their ecological function.

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Phytotoxicity of Antofine from Invasive Swallow-Worts

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Abstract Pale swallow-wort (*Vincetoxicum rossicum*) and black swallow-wort (*V. nigrum*) are two emerging invasive plant species in the northeastern United States and southeastern Canada that have shown rapid population expansion over the past 20 years. Using bioassay-guided fractionation, the known phytochemical phenanthroindolizidine alkaloid, (−)-antofine, was identified as a potent phytotoxin in roots, leaves, and seeds of both swallow-wort species. In seedling bioassays, (−)-antofine, at μM concentrations, resulted in greatly reduced root growth of *Asclepias tuberosa, A. syriaca,* and *Apocynum cannabinum*, three related, native plant species typically found in habitats where large stands of swallow-wort are present. In contrast, antofine exhibited moderate activity against lettuce, and it had little effect on germination and root growth of either black or pale swallow-wort. In disk diffusion assays, antifungal activity was observed at 10 μg and 100 μg, while antibacterial activity was seen only at the higher level. Although both swallow-wort species display multiple growth and reproductive characteristics that may play an important role in their invasiveness, the presence of the highly bioactive phytochemical (−)-antofine in root and seed tissues indicates a potential allelopathic role in swallow-worts' invasiveness.

Key Words Antofine · Phytotoxin · Swallow-worts · *Vincetoxicum* · *Cynanchum* · Allelopathy · Invasiveness · Allelopathic potential

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Introduction

Two emerging invasive plant species in the northeastern United States and southeastern Canada are pale swallow-wort [*Vincetoxicum rossicum* (Kleopow) Barbarich = *Cynanchum rossicum* (Kleopow) Borhidi] and black swallow−wort [*V. nigrum* (L.) Moench = *C. louiseae* Kartesz & Gandhi]. Both exist as herbaceous, perennial, twining vines and are related to milkweeds (Apocynaceae, subfamily Asclepiadoideae). Pale swallow-wort is native to the Ukraine and European Russia and possesses pink to maroon flowers, whereas black swallow-wort is native to southwestern Europe and bears dark purple to black flowers (Markgraf, 1972). These species were introduced into North America, probably as ornamentals, in the mid- to late-1800s, but not until the 1970's were concerns raised about the invasive potential of swallow-worts (Pringle, 1973). The last 20 years have seen a rapid expansion of both species (Lawler, 2000; DiTommaso et al., 2005b), which are currently reported in 21 U.S. states from New England to Maryland and west to Nebraska and Kansas, with an additional report from California, and the Canadian provinces of Ontario and Quebec (Kartesz, 1999; DiTommaso et al., 2005b; USDA 2009). The primary infestations are in New England, New York, and Ontario, and both species are listed as noxious weeds or prohibited invasive species in Vermont, New Hampshire, Massachusetts, and Connecticut (USDA 2009).

Both pale and black swallow-wort have multiple growth and reproductive characteristics that may play a role in their invasiveness. Swallow-wort vines can grow 1–2 m or more during the growing season, producing dense, tangled masses of vines that climb up and over supporting vegetation (Christensen, 1998; Douglass et al., 2009). Stand densities can average 134 stems per m^2 and 1800

seedlings per m^2 (Smith et al., 2006). Large stands reportedly exclude most other vegetation, including the related milkweeds, and are encroaching on at least five threatened or endangered species in New York and New England and on the globally-rare alvar communities in Ontario and New York (Haribal and Renwick, 1998; Lawler, 2000; Tewksbury et al., 2002; Douglass et al., 2009). Perennating buds produced on the rootstock give rise to one or more herbaceous stems in the spring, and additional buds will sprout if stems are damaged (DiTommaso et al., 2005b). Flowering is indeterminate, extending from mid-May into August, and seeds bear an apical tuft of hairs, which aids in long-distance dispersal (Ladd and Cappuccino, 2005). Both swallow-wort species are self-compatible and can self-pollinate or are cross-pollinated by insects (Lumer and Yost, 1995; St. Denis and Cappuccino, 2004). Seed production can be prolific in high-light environments—around 32,000 seeds per m^2 for high density stands of pale swallow-wort, and seeds are polyembryonic, containing 1–8 embryos (Cappuccino, 2004; DiTommaso et al., 2005a; Smith et al., 2006). Polyembryony may contribute to enhanced seedling establishment and survival rates, which can be over 50% for pale swallow-wort (DiTommaso et al., 2005a; Ladd and Cappuccino, 2005; Hotchkiss et al., 2008). Both species are drought tolerant and can grow in soils of a wide range of pH (DiTommaso et al., 2005b; Magidow et al., 2008). Pale swallow-wort also is shade tolerant, although in heavily shaded areas, little reproduction by seed occurs (Smith et al., 2006; Hotchkiss et al., 2008).

What is not clear is whether allelopathy also plays a role in the invasiveness of swallow-wort. Members of the Apocynaceae (including the Asclepiadoideae) are regarded as poisonous to humans and other mammals (Wiegrebe et al., 1970; Haznagy and Toth, 1973; Haeggstrom, 1990). In North America, swallow-wort plants sustain minimal damage from herbivory, as deer rarely browse them and few arthropod herbivores feed on them (Christensen, 1998; DiTommaso et al., 2005b; Ernst and Cappuccino, 2005; Milbrath, 2010).

Phenanthroindolizidine alkaloids, primarily (−)-antofine and antofine oxide, as well as several less abundant derivatives, have been isolated from the roots and aerial parts of *V. hirundinaria* Medik. (Wiegrebe et al., 1969; Lavault et al., 1994; Staerk et al., 2000; Staerk et al., 2002). (−)-Antofine also has been reported from root tissues of *V. pumilum* Dcne. (Staerk et al., 2005) and the aerial parts of black swallow-wort (Capo and Saa, 1989). (−)-Antofine and its derivatives, including antofine oxide, possess antifungal, antibacterial, and cytotoxic activity due to potent inhibition of protein and nucleic acid synthesis and G2/M cell cycle arrest (Ferenczy et al., 1965/1966; Rao and Venkatachalam, 2000; Staerk et al., 2002; LEE et al., 2003). Root extracts of pale swallow-wort have phytotoxic and antifungal properties (Cappuccino, 2004); antofine was identified as the antimicrobial agent, while the component(s) responsible for phytotoxic or antifeedant activity has not been identified (Mogg et al., 2008). Similar phytotoxic effects of both root exudates and tissue leachates from pale and black swallow-wort on lettuce and butterfly milkweed root growth recently have been reported (Douglass et al., 2011).

We undertook this study to determine if specific phytochemical constituents from the swallow-worts possess phytotoxic activity, a necessary step to evaluate whether allelopathy could play a role in swallow-worts' ability to displace native plant species in the environment. We herein report that the known phytochemical antofine is present in root, shoot, and leaf, and seed extracts of both pale and black swallow-wort, and that it has significant phytotoxicity against some related native plant species with little effect against pale or black swallow-wort germination and root development.

Methods and Materials

Plant Material Five plants of pale swallow-wort were excavated from Robert G. Wehle State Park, Jefferson Co., New York, in May 2005, and placed into a cooler for transport to the laboratory where the roots (with stems attached) were rinsed in several volumes of water to remove remaining soil. After removal of the stem and crown area, roots were weighed, frozen at −20°C, and then lyophilized to dryness and stored at −20°C until extracted. Rootstocks of black swallow-wort were excavated from Bear Mountain State Park, Rockland Co., New York, in September 2005. They were held under refrigeration until January 2006 and then grown in a greenhouse for 4 mo until being harvested and prepared as noted above. For seed extractions, pale swallow-wort seed was collected in August 2008 from fields in Great Gully Preserve, (Cayuga Co.), New York, and black swallow-wort seed was collected in August 2008 from fields in Bear Mountain State Park, (Rockland Co.), New York. After pods were dried and crushed to release seeds, the majority of pod and pappus materials were removed by hand vacuum, and then seeds were sieved using mesh sizes of 30/64″, 25/64″, 19/64″, 15/64″, 11/64″, 9/64″, 7/64″, and 5/64.″ Filled pale swallow-wort seed was collected on the 7/64″ sieve, while filled black swallow-wort seed was collected on the 11/64″ and 9/64″ sieves. Cleaned seeds were stored at 4°C until use.

Seed Collections for Laboratory Bioassays For initial tests of phytotoxicity from crude swallow-wort extracts, seeds were obtained from the following sources: lettuce (*Lactuca sativa* L. cv. black-seeded Simpson) from a local gardener's supply store; black swallow-wort, Bear Mountain State

Park, Rockland Co., New York, summer 2005; pale swallow-wort, Great Gully Preserve, Cayuga Co., New York, summer 2005; *Asclepias syriaca* L. (common milkweed), Great Gully Preserve, Cayuga Co., New York, fall 2005; *Asclepias tuberosa* L. (butterfly milkweed), Clark Reservation State Park, Jamesville, Onondaga Co., New York, fall 2005; *Apocynum cannabinum* L. (Indianhemp), Great Gully Preserve, Cayuga Co., New York, fall 2005.

For phytotoxicity tests with purified antofine, seeds were obtained from the following sources: black swallow-wort, Bear Mountain State Park, Rockland Co., New York, fall 2008; pale swallow–wort, Great Gully Preserve, Cayuga Co., New York, fall 2008; *A. syriaca*, Everwilde Farms, Inc., Bloomer, WI, USA; *A. tuberosa*, Everwilde Farms, Inc., Bloomer, WI, USA; *A. cannabinum*, Great Gully Preserve, Cayuga Co., New York, fall 2008.

All seed, except lettuce, was air-dried, cleaned, and stored dry in sealed containers under refrigeration (4°C) for approximately 2 mo prior to stratification. To stratify seed for bioassays, approximately 5 g of seeds were wrapped in moist, brown paper toweling, and then stored in sealed glass jars with rubber gaskets or sealed petri dishes for 2–3 mo at 4°C.

Seedling Root Growth Bioassay Solutions were applied in 1.0 ml methanol to evenly coat the bottom of 10×10 cm square Petri plates. After solvent evaporation (30 min), 20 ml of 1.6% agarose were pipetted into each plate, agitated briefly by hand, and set to cool for 1 h at 22°C; tested concentrations are given as amount/ml agarose. Seeds were located in a row of 15 on the surface of each plate ca. 20 mm from the "top" edge of the plate with the root-ends all pointed in the same direction. Plates were covered, sealed with parafilm, and stored upright (with root-ends pointed down) in a chamber with a thermoperiod of 25:20°C and photoperiod of 14:10 hL:D. Root lengths were measured typically at 7 and 14 d. Two plates of 15 seeds each were used for each dosage of each compound, with each assay independently replicated at least twice. Measurements were averaged for each level, and % root length was calculated relative to the average root length observed on the methanol only control –treated plates.

Extraction of Swallow-Wort Plant Materials For initial phytotoxicity testing, approximately 10 g either of freeze-dried roots or shoot/leaf material of pale and black swallow-wort were sequentially extracted with hexane, dichloromethane, ethanol, and water. Each fraction was dried, and then reconstituted in methanol at 40 mg/ml and assayed for phytoxicity against lettuce seeds. For larger scale preparations and for chemical analysis, freeze-dried plant materials were extracted with 10 vol (v/wt) of dichloromethane:methanol (1:1). After gentle stirring for 24 h, the solvent was removed by filtration, and the residual powder was re-extracted for an additional 24 h with 10 vol (v/wt) of dichloromethane:methanol (1:1), treated as above, and then both extracts were combined, and dried *in vacuo* by rotary evaporation. Seed samples (2 g per replicate) were ground to a fine powder in a coffee mill, and then extracted as above. Extracts were combined and filtered through 2 layers of Whatman No. 1 filter paper and then dried *in vacuo* by rotary evaporation.

Preparative TLC Silica plates (Baker-500 μM thickness; 25×25 cm), loaded with 6 mg of pale swallow-wort extract, were used to preparatively separate the constituents of the active root fractions, using ethyl acetate:methanol (60:40) as a developing solvent. Bands observed under short and long-wave UV were recombined into seven fractions by scraping and elution in MeOH. Fractions were reanalyzed (Baker-40-μm; 25×0.5 cm) for confirmation of Rf prior to testing for phytotoxicitiy against lettuce seedlings.

Purification of Active Phytotoxic Fraction Freeze-dried root material (approximately 50 g) was ground to a fine powder in a coffee mill, and then extracted in dichloromethane:methanol (1:1) as described above to yield 3.3 g of a brownish-yellow gum. The crude extract then was partitioned between dichloromethane (1 vol) and water (2 vol), the organic layer was dried over anhydrous Na_2SO_4, and the solvent removed *in vacuo* to afford an oily yellowish-brown residue (0.76 g) that retained all of the original activity of the crude extract. This material was dissolved in dichloromethane and chromatographed on silica gel (Baker 40-μm; 300×25 mm), eluted with 400 ml each of 100% dichloromethane, 1, 2, 5, 10, 20, and 50% methanol in dichloromethane, and 100% methanol. The 10% MeOH fraction, which exhibited the greatest phyotoxic activity, was re-chromatographed on silica column (Baker-40-μm; 25×1 cm) eluted with 50 ml 100% dichloromethane, 200 ml each of 10%, 20%, and 50% methanol in dichloromethane. Phytotoxic activity was recovered in the 10% methanol fraction that afforded (−)-antofine (approx. 90%)(22.3 mg). At each step in the purification, fractions were monitored using TLC on silica with ethyl acetate:methanol (60:40) as the mobile phase. Semi-preparative HPLC was used to obtain purified material for spectral analysis.

Antimicrobial Bioassay **(−)-**antofine was tested in a disk diffusion assay against bacterial strains (*Escherichia coli, Bacillus cereus,* and *Bacillus subtilis*) grown on nutrient agar plates (9 cm) and against fungal target strains (*Colletotrichum acutatum, Fusarium graminearum, Beauveria bassiana,* and *Metarhizium anisopliae*) grown on potato

dextrose agar plates. Plates spread with 0.5 ml aliquots of a liquid bacterial culture grown for 24 h in nutrient broth or with 0.5 ml of a fungal spore suspension (1×10^6 spores/ml) were allowed to dry for 1 h. Test samples were applied to 4-mm filter paper disks in 10 μl methanol, air-dried for 1 h, and then placed on test plates. Zones of inhibition were measured after 48 h. Antofine was tested at 10 and 100 μg/disk. Methanol was used as a negative control (10 μl); positive controls used were iturin (10 μg) or filipin (10 μg) (Sigma) applied in methanol for fungal assays and commercial disks (Sigma) pretreated with ampicillin (10 μg) or erythromycin (15 μg) for bacterial assays.

HPLC Analysis Analytical HPLC was carried out by a modification of the method of Cui et al. (2004) employing a reversed phase C18 column (Varian, 5 μm, 250×4.6 mm), premixed isocratic mobile phase of methanol: 20 mM ammonium acetate (1:1 v/v), flow rate of 1 ml/min, with primary detection by UV at 260 nm and a wavelength scan of 200 to 350 nm. Retention time (RT) of antofine under these conditions was 6.5 min, with limit of detection (s/n=10) established at 0.2 μg on-column. The peak corresponding to antofine oxide (RT=6.0 min) possessed an almost identical photo diode array (PDA) spectrum and was quantified using peak area equivalences to antofine. Semi-preparative HPLC was carried out using a (ODS)3 column (end capped C18, Phenomenex, 5 μm, 250×4.6 mm), premixed isocratic mobile phase of methanol: 20 mM ammonium acetate (3:2 v/v), flow rate of 1 ml/min, with primary detection by UV at 260 nm and a wavelength scan of 200 to 350 nm, that allowed longer retention times for peak collection of the phytotoxic components.

Spectrometric Measurements Low-resolution electrospray ionization mass spectra (LRESIMS) spectra were acquired by infusion of methanolic solutions at 5 μl/min by a syringe pump (Harvard apparatus) into a Micromass ZMD-4000 spectrometer. Positive-ion-mode spectra were acquired with capillary cone voltages of 3.2 kV and 40 V, respectively; negative-ion-mode spectra were acquired with capillary and cone voltages of 4.0 kVand 20 V, respectively. High-resolution electrospray ionization mass spectrometry (HRESIMS) data were acquired by the mass spectrometry facility at the University of Illinois, Urbana Campus, USA.

Statistical Analysis Data was analyzed using JMP® statistical software, version 8.0.1; Tukey-Kramer's *post-hoc* test ($P<0.05$) was used for comparisons within treatments of solvent-tissue extracts and for initial effects on plant species. Dose–response curves were fit to a 4-parameter logistic model (Streibig et al., 1993), and I_{50} values were estimated from these curves.

Results

Concentration-dependent Phytotoxicity of Swallow-wort Extracts Sequential extraction of root and combined leaf and shoot materials from pale swallow-wort followed by bioassay testing using lettuce seedlings at 0.5 mg/ml applied dose revealed that the phytotoxic activity resided primarily in the dichloromethane and ethanol fractions (Fig. 1), which represented 1.5% and 2.5%, respectively, of the root dry weight, and 1.8% and 3.8%, respectively, of the dry weight of the shoot/leaf material. For further analysis and subsequent purification of the phytotoxic principles, both pale and black swallow-wort root tissue were extracted with dichloromethane: methanol (1:1). To assess activity against seed germination and root growth of native plant species, extracts were tested at 0.25 mg/ml and 1 mg/ml. Inhibitory activity of pale swallow-wort extract was evident at both levels against a number of native plant species that co-occur with invasive swallow-worts (Fig. 2). Black swallow-wort extract was not as potent, showing activity only at the 1 mg/ml level (data not shown). Little activity was noted with either extract, when tested against itself or the congeneric species (Fig. 2).

Identification of the Phytotoxic Constitutent of Pale and Black Swallow-Wort

Phytotoxic activity was confined to a single TLC band (Rf=.62) visible as a dark spot under long wave UV that represented less than 1% of the original extract. Combined

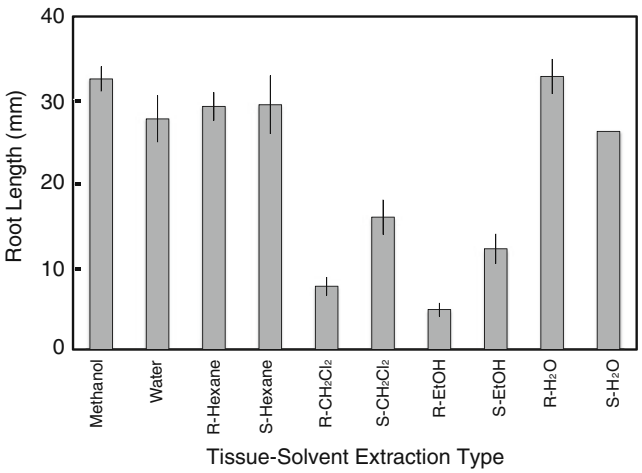


Fig. 1 Effect of sequential extractions of root and shoot/leaf tissues of pale swallow-wort on lettuce seedling root length. Measurements were taken at 1 wk of growth with a thermoperiod of 25:20°C and photoperiod of 14:8 hL:D using methanol and water as negative controls with 0.5 mg/ml applied dose of each extract. Data plotted are the mean ± SE, ($N=30$). R = root tissue; S = shoot and leaf tissue

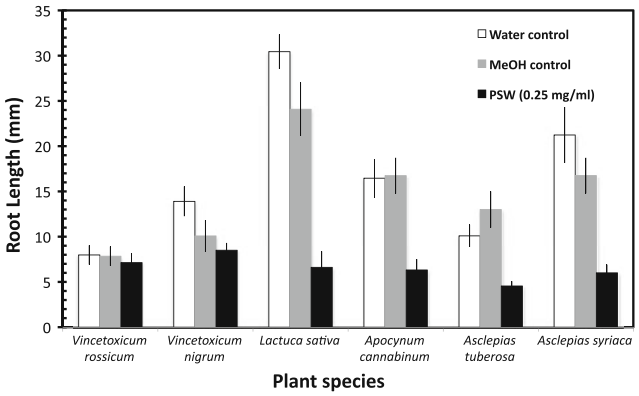


Fig. 2 Effect of pale swallow-wort (PSW) root extract at 0.25 mg/ml applied dose on root lengths of plant seedlings measured at 1 wk. Plates were incubated with a thermoperiod of 25:20°C and photoperiod of 14:8 hL:D. Data plotted are the mean ± SE, (N= 30)

fractions from silica gel chromatography were monitored by dose equivalence bioassay and by analytical TLC to monitor the elution of the major phytotoxic principle. Phytotoxic activity was recovered in the 10% methanol:dichloromethane fraction, representing 0.05% dry wt of the starting material.

LRESIMS analyses of the phytotoxic fraction indicated the presence of a major component with a molecular weight of *m/z* [M+H]+= 364, which is consistent with the major phenanthroindolizidine alkaloid, (−)-antofine, previously identified from shoot tissues of *V. rossicum* and *V. nigrum* and related *Vincetoxicum* species (Wiegrebe et al., 1970; Capo and Saa, 1989; Staerk et al., 2000, 2005; LEE et al., 2003), and recently reported from root extracts of *V. rossicum* (Mogg et al., 2008). Semi-preparative HPLC was used to collect one minor (RT=6.5 min) and two major peaks (RTs=8.0 and 13.5 min), all of which had elution and UV spectral characteristics consistent with published values for antofine (Wiegrebe et al., 1969; Cui et al., 2004). The minor peak was putatively identified as hydroxy antofine oxide based on LRESIMS of *m/z* [M+H]+= 396, and was not examined further. HRESIMS data for the major component eluting at RT=13.5 min was consistent with the molecular formula $C_{23}H_{26}NO_3$ (for $[M+H]^+$, obsd *m/z* 364.1911; calcd 364.1913). The component eluting at RT=8.0 min, with a molecular weight of *m/z* [M+H]+= 380 on LRESIMS, was consistent with antofine oxide, another known compound reported from *Vincetoxicum* species. HRESIMS data for this component was consistent with the molecular formula $C_{23}H_{26}NO_4$ (for $[M+H]^+$, obsd *m/z* 380.1860; calcd 380.1862). The purified active fraction represented 0.05% (dry wt basis) from pale swallow-wort roots and 0.015% from black swallow-wort roots, similar to concentrations of antofine reported from *V. pumilum* and *V. rossicum* root tissue (Staerk et al., 2005; Mogg et al., 2008).

Phytochemical Activity of Antofine Activity of the antofine preparation was tested over a concentration range from 0.1 to 75 μg/ml using the seedling bioassay system. Root growth of all tested plant species was inhibited by antofine to some degree, but differential sensitivity to the phytotoxin was evident when activity was tested against three native plant species. Little inhibition of root growth of pale and black swallow-wort seedlings was seen up to a concentration of 75 μg/ml, while lettuce was moderately affected, producing an I_{50}=12.5 μg/ml (34 μM) (Fig. 3). In contrast, root growth of *A. tuberosa* and *A. syriaca* was markedly inhibited by antofine, with I_{50}=1.25 and 3.1 μg/ml (3.4 and 8.6 μM), respectively (Fig. 4). *Apocynum cannabinum* root growth also was inhibited but at slightly higher concentrations (I_{50}= 3.75 μg/ml; 10.3 μM). Extensive browning of the root tip was visible on all of these native species, with little to no growth by 25 μg/ml (69 μM). In bioassays against pale or black swallow-wort, root growth was only slightly reduced at the highest concentration of 75 μg/ml (206 μM) with little visible tissue damage.

Detection of (−)-Antofine in Seeds of Pale and Black Swallow-Wort HPLC and LRESIMS data indicated that antofine was present in seeds of both black and pale swallow-wort seeds, a previously undocumented source of phenanthroindolizidine alkaloids, which have been reported only from roots and aerial portions of *Vincetoxicum.* As assessed by HPLC, antofine was detectable at concentrations of 1.10% (fresh wt) and 0.45% in seeds of pale and black swallow-wort, respectively.

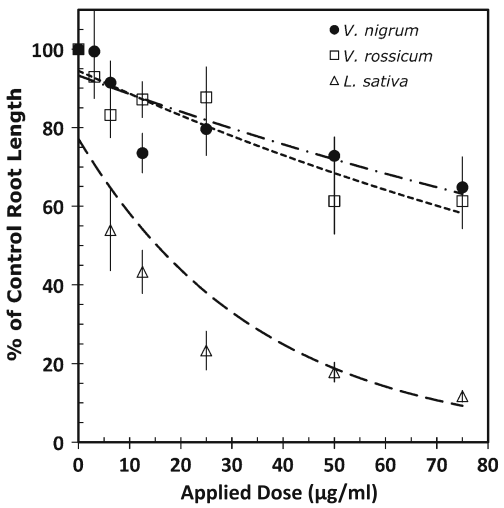


Fig. 3 Dose response profiles, plotted as a percentage (%) of the control root length in the absence of antofine, of *Vincetoxicum nigrum, V. rossicum*, and *Lactuca sativa* seeds germinated in the presence of varying concentrations of antofine. Plates were incubated with a thermoperiod of 25:20°C and photoperiod of 14:8 hL:D, and scored after 14 d. Data plotted are the mean ± SE, (N= 30)

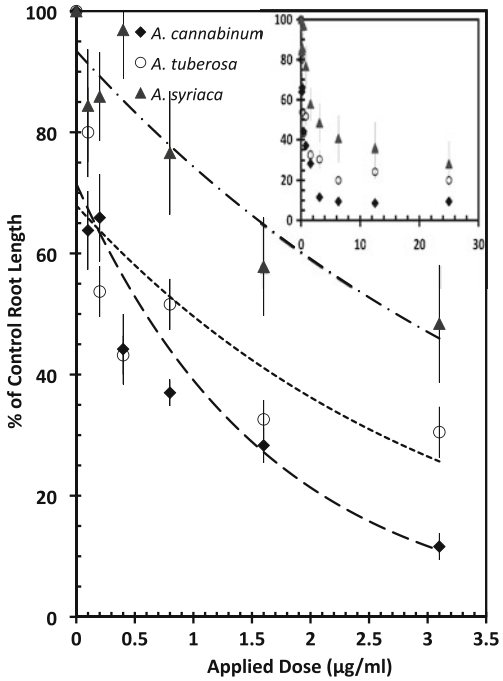


Fig. 4 Dose response profiles, plotted as a percentage (%) of the control root length in the absence of antofine, of *Apocynum cannabinum, Asclepias tuberosa, and A. syriaca* seeds germinated in the presence of varying concentrations of antofine. Plates were incubated with a thermoperiod of 25:20°C and photoperiod of 14:8 hL:D, and scored after 14 d. Inset shows response under an expanded range of concentrations, with little/no root growth evident at concentrations >25 µg/ml applied dose. Data plotted are the mean ± SE, (N= 30)

Antibiotic Activity At 48 h, clear zones of antifungal activity were evident from the 100 µg antofine treatment similar to that observed with the positive controls (Table 1). Much smaller zones were visible with the 10 µg antofine treatment, at levels consistent with previous reports (Baumgartner et al., 1990; Mogg et al., 2008). The antibacterial activity was less pronounced at the same concentrations; no activity was observed with 10 µg antofine, while only small clear zones of inhibition (2 mm) were observed at the higher level in comparison to the large kill zones from all positive controls (Table 1).

Discussion

We undertook the current study to assess the potential contribution of phytotoxins to swallow-worts' invasiveness through allelochemical interactions. Among the many hypotheses advanced to understand the dynamics of invasive species, the "novel weapons" or "allelopathic advantage against resident species" (AARS) hypothesis (Bais et al., 2003; Callaway and Ridenour, 2004) is based on the production of allelochemicals in sufficient quantities by the invasive species to produce phytotoxicity in a group of susceptible, or non-adapted, plants. This hypothesis was originally based on a series of observations that *Centauraea diffusa* and *C. maculosa* were at a competitive advantage relative to native species in the invaded range compared to their native range (Callaway and Aschehoug, 2000; Bais et al., 2003). Debate about this hypothesis continues because of conflicting estimates of (±) catechin levels from *Centaurea* species in soils that call into question whether concentrations are sufficient to cause phytotoxicity (Blair et al., 2005). Much of the original work has been discredited

Table 1 antimicrobial activity of (−)-antofine

Test Organism				
	Metarhizium acridum	*Beauveria bassiana*	*Fusarium graminearum*	*Colletotrichum acutatum*
Treatment			Inhibition Zone (mm)	
– (−) antofine				
10 µg	5	3	0	1
100 µg	12	6	1	5
Methanol −10 µl	0	0	0	0
iturin −10 µg	nt	4	nt	nt
filipin −10 µg	4	nt	5	5
	Escherichia coli	*Bacillus cereus*	*Bacillus subtilis*	
–(−) antofine				
10 µg	0	0	0	
100 µg	2	2	0	
methanol −10 µl	0	0	0	
ampicillin −10 µg	3	2	4	
erythromycin −15 µg	8	5	11	

nt not tested

(see Blair et al., 2006; Norton et al., 2008; Duke et al., 2009); at least one paper has been withdrawn (Weir et al., 2003) and soil and exudate concentrations have been revised (Weir et al., 2009; Bais et al., 2010), leaving questions as to whether allelochemicals can contribute to invasiveness. Recent field evidence has indicated that root exudates containing (±) catechin are allelopathic against species that co-occur in the invaded range, but do not cause a similar inhibitory effect in the native range (Thorpe et al., 2009). Some of these difficulties may be due to experimental problems with detection, extraction from various soil types, the possibility of pulsed releases, and stability of the compound in the soil (Inderjit et al., 2008a). The novel weapons hypothesis continues to be attractive given the numerous examples of allelopathic effects of exudates (Hierro and Callaway, 2003; Inderjit et al., 2008b) and the presence of novel chemistries in invasive exotic plants (Cappuccino and Arnason, 2006), although few chemicals have been tied specifically to an environmentally relevant allelopathic trait associated with invasiveness.

Previous observations have led to the suggestion that swallow-worts might affect rhizosphere dynamics through the production of allelochemicals. Shifts in arbuscular mycorrhizal fungi that benefit swallow-wort growth have been detected (Greipsson and DiTommaso, 2006; Smith et al., 2008). When evaluating potential Allee effects (latency in the rate of spread) in *V. rossicum* populations, Cappuccino (2004) noted the greater suppression of background vegetation by larger patches relative to smaller plant treatments. Others have suggested that purported allelochemicals could play a role in competitive plant interactions (DiTommaso et al., 2005b). Antofine was identified in *V. rossicum* root extracts and shown to have broad-spectrum antifungal activity (Mogg et al., 2008), but anti-feedant activity against sawfly larvae and masked birch caterpillar was attributed to an as-yet unidentified compound. Allelopathic activity from *V. rossicum* has been reported, but the chemistry involved was not identified (Cappuccino, 2004; Douglass et al., 2011).

This study extends the spectrum of antofine's bioactivity to include phytotoxicity which is especially potent against native asclepiads. We attributed phytotoxicity of root extracts of pale and black swallow-worts to the presence of (−)-antofine, an antimicrobial and cytotoxic phenanthroindolizidine alkaloid previously reported from *Vincetoxicum* species. The concentration of antofine in pale and black swallow-wort extracts correlates with the level of crude extract required for biological activity in seedling bioassays; in our preparations, pale swallow-wort root tissues had approximately four times the level of that found in black swallow-wort roots. We also documented antifungal and antibacterial activity of antofine at submilligram levels as shown previously, but we discovered that antofine also was biologically active at μM concentrations in seedling bioassays. Antofine demonstrated potent phytoxicity against a few native North American plant species that typically are found in areas being invaded by the non-native swallow-worts, although there was little activity seen when tested against either pale or black swallow-wort. We also report the presence of antofine in seeds, at concentrations approximately 2–3 times higher than that detected in roots or aerial portions of the plant. All of these findings point to antofine, and perhaps antofine oxide and other closely related alkaloids also reported to have cytotoxic activity (Staerk et al., 2000, 2002, 2005), as potential candidate phytochemicals that could exhibit allelopathic effects on surrounding vegetation and rhizosphere dynamics. Our preliminary experiments using antofine-spiked soil indicate that antofine is capable of causing root growth inhibition but at higher concentrations than found in the seedling bioassay system. We anticipate that soil type and pH will affect adsorption, stability, and availability of antofine as a potential allelochemical, so experiments are underway to determine antofine concentrations in undisturbed rhizospheres as well as the efffective recovery rates as a function of soil type and extraction method.

Invasive plants typically exhibit a suite of attributes that collectively contribute to their invasiveness. In the case of the swallow-worts, their success as invasive weeds in the northeastern U.S. may be due, at least in part, to their high fecundity (Smith et al., 2006) and their ability to adapt to a variety of habitats (Sheeley and Raynal, 1996; DiTommaso et al., 2005b), leading to an displacement of native species within native and disturbed environments (Ladd and Cappuccino, 2005; Douglass et al., 2009). The disproportionate production of root biomass relative to aerial plant development in pale swallow-wort would provide significant advantages for obtaining nutrients (Cappuccino, 2004; Smith et al., 2006; Milbrath, 2008); once established in satellite populations, the plants exhibit profuse and aggressive growth that can alter the light canopy, among other environmental factors (DiTommaso et al., 2005b). Whether this active growth phase also involves allelochemical interactions that aid in the displacement of native populations is as yet unproven. This study provides laboratory evidence that the phytochemical properties of antofine could contribute to swallow-worts' invasiveness in non-native habitats. Future studies are needed to determine whether antofine and related compounds are present at sufficient levels within the soil and rhizosphere to cause physiological responses relevant to invasion ecology of pale and black swallow-wort populations.

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Effects of a Coumarin Derivative, 4-Methylumbelliferone, on Seed Germination and Seedling Establishment in *Arabidopsis*

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Abstract The root system is central for plant adaptation to soil heterogeneity and is organized primarily by root branching. To search for compounds that regulate root branching, a forward chemical genetics screen was employed, and 4-methylumbelliferone (4-MU), a coumarin derivative, was found to be a potent regulator of lateral root formation. Exogenous application of 4-MU to *Arabidopsis thaliana* seeds affected germination and led to reduced primary root growth, the formation of bulbous root hairs, and irregular detached root caps accompanied by reorganization of the actin cytoskeleton in root tips before seedling establishment. Abundant lateral roots formed after exposure to 125 μM 4-MU for 22 days. Molecular, biochemical, and phytochemical approaches were used to determine the effect of 4-MU on root growth and root branching. *Arabidopsis* seedlings grown in the presence of 4-MU accumulated this compound only in roots, where it was partially transformed by UDP-glycosyltransferases (UGTs) into 4-methylumbelliferyl-β-D-glucoside (4-MU-Glc). The presence of 4-MU-Glc in seedling roots was consistent with the upregulation of several genes that encode UGTs in the roots. This shows that UGTs play an integral role in the detoxification of 4-MU in plants. The increased expression of two auxin efflux facilitator genes (*PIN2* and *PIN3*) in response to 4-MU and the lack of response of the auxin receptor *TIR1* and the key auxin biosynthetic gene *YUCCA1* suggest that auxin redistribution, rather than auxin biosynthesis, may directly or indirectly mediate 4-MU-induced root branching.

Key Words Coumarin · 4-methylumbelliferone · Glycosyltransferases · Detoxification · Lateral root

Introduction

Plants are sessile and must endure environmental challenges, which include changes in water and nutrient availability and their heterogenous distribution in the soil. To acquire water and nutrients efficiently, plants dynamically modulate their root systems by regulating primary root growth, lateral root formation, and root hair number (de Dorlodot et al., 2007; Osmont et al., 2007; Nibau et al., 2008). Although progress has been made in dissecting the hormonal, genetic, and nutritional control of lateral root development (de Dorlodot et al., 2007; Osmont et al., 2007; Nibau et al., 2008; Peret et al., 2009), the mechanisms underlying the initiation and emergence of lateral roots are unclear. The formation of lateral roots is dependent on the coordinated action of many factors. In *Arabidopsis thaliana*, more than seventy genes have been shown to influence lateral root development (De Smet et al., 2006); however, the plant hormone auxin and its polar transport and redistribution have emerged as key factors (Fukaki et al., 2002; Nibau et al., 2008; Peret et al., 2009; Vanneste and Friml, 2009). Intercellular auxin movement is controlled by several plant-specific pin-formed (PIN) proteins that facilitate auxin efflux (Vanneste and Friml, 2009).

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Coumarins (1, 2-benzopyrones) are secondary metabolites often found in roots of higher plants where they originate from the general phenylpropanoid pathway (Bourgaud et al., 2006). While their exact biological function in plants remains unclear, historically they are known to be involved in plant defense (Baumert et al., 2001; Carpinella et al., 2005; Brooker et al., 2007) along with flavonoids, phenolics, and alkaloids. Unlike most other secondary metabolites, coumarins appear to specifically inhibit seed germination and root growth (Svensson, 1971; Abenavoli et al., 2001, 2004, 2006, 2008; DeBolt et al., 2007), and this is consistent with previous observations that coumarins accumulate mainly in roots. They also influence membrane transport in the root tip (Lupini et al., 2010) and nitrogen uptake and metabolism (Abenavoli et al., 2001, 2003). Some natural coumarins also have received attention for their pharmacological properties, such as inhibition of HIV-1 and in treating thromboembolic diseases (Kostova et al., 2006).

Chemical genetics has been used extensively in plant biology for the identification of metabolites and their candidate genes involved in specific biochemical pathways (Blackwell and Zhao, 2003). As part of our efforts to characterize natural products that affect seed germination, root growth, and root architecture, we used forward chemical genetics to screen a small-molecule chemical library. Here, we focused on a coumarin derivative, 4-methylumbelliferone (4-MU), which inhibited germination and primary root growth, but promoted root branching after seedling establishment. We investigated the role of this compound on these parameters in more detail and demonstrated that 4-MU specifically accumulates in roots, where it is transformed into 4-methylumbelliferyl-β-D-glucoside (4-MU-Glc) by the action of UDP-glycosyltransferases (UGTs). We propose that UGTs play an integral role in the biochemical detoxification of 4-MU in *Arabidopsis* during seed germination and seedling development.

Methods and Materials

Plants, Chemicals and Forward Chemical Genetic Screens All chemical standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents used were high-pressure liquid chromatography (HPLC) grade. *Arabidopsis thaliana* ecotype Columbia (Col-0) was obtained from the *Arabidopsis* Biological Resource Center (Columbus OH, USA). Seeds were cold-treated at 4°C for 2 d before germination. A collection of 128 natural compounds was screened by germinating and growing *Arabidopsis* on sterile Murashige and Skoog (MS) medium (Sigma) supplemented with 1.2% agarose and 0, 25, 50, or 125 μM compound. Germination and seedling growth were conducted in a growth cabinet with 16 hr of light (22°C), 8 hr dark (18°C) and 60% relative humidity. Plants were grown for different lengths of time in 4-MU MS media based on the objectives of the experiments. For observation of 4-MU effects on seed germination and primary root growth, plants were treated for 6 d. For auxin transport analysis and MUG assays in root systems, plants were harvested after treatment for 14 d when lateral root branching has been initiated and *DR5::GUS* was actively expressed (Supplementary Data Fig. 1). To measure the effect of 4-MU on seedling establishment and root architecture, plants were treated for up to 22 d when seedlings and root branching were well established. Fresh weight and root length were measured immediately after harvest. The *35S::ABD-GFP* (Motes et al., 2005) construct was provided by Dr. Elison B. Blancaflor, and the *DR5::GUS* transgenic line (Ulmasov et al., 1997) by Dr. Tom Guilfoyle (University of Missouri).

GUS Expression Analysis Quantitative analysis of GUS activity was conducted using 4-methylumbelliferyl β-D-glucuronide hydrate (MUG) fluorometric assays (Jefferson, 1987). Seedlings (200 mg) were ground with 1 ml of GUS extraction buffer containing 0.1 M Na_2HPO_4 (pH 7.5), 10 mM EDTA, 0.1% Triton X-100, and 10 mM β-mercaptoethanol at 4°C. After centrifugation, 100 μl of the extract were incubated with 2 mM MUG in extraction buffer in a final volume of 1 ml for 3 hr at 37°C. Three independent replicates were conducted.

Fluorescent Confocal Microscopy Laser scanning confocal and differential interference contrast microscopy was performed with a Carl Zeiss LSM150 microscope (Department of Veterinary Biomedical Sciences, University of Saskatchewan). GFP fluorescence was detected using excitation and emission wavelengths of 488 and 505 nm, respectively. The data shown are representative of three independent experiments. The images were analyzed using NIH Image J 1.37 software (http://rsb.info.nih.gov/ij/).

Identification and Purification of 4-MU-O-β-D-Glucopyranoside Arabidopsis roots were harvested and frozen at −80°C from plants grown 6 d either in the presence or absence of 4-MU. A 100 mg sample of frozen roots was ground in liquid nitrogen in a 20 ml Potter-Elvehjem, followed by grinding in 10 ml methanol/water (80:20; v/v) for 10 min. After filtration, the pellet was extracted again with methanol/water overnight at 4°C in the dark. The two extracts were combined and dried at 35°C under vacuum, and the dried extract was resuspended at 1 mg ml^{-1} with methanol/water (50:50; v/v). HPLC/UV analysis was conducted using a Hewlett Packard Agilent 1100 chromatograph, a G-7120 diode array detector, HP Chemstation ver. 08.01 software and a Zorbax C_{18} column

(150×4.6 mm, 5 μm, Mississauga, ON, Canada) with a 60 min linear gradient of 5–75% methanol v/v, a flow rate of 1.0 ml min^{-1}, and an injection volume of 2 μl. LC-MS profiles were obtained on an Agilent 1100 HPLC and an API QStar XL pulsar hybrid system (Applied Biosystems, Foster City, CA, USA) (Li et al., 2011).

Identical chromatography conditions were used for semi-preparative purification of 4-MU-*β*-D-glucoside (4-MU-Glc) from 5 g of 4-MU-treated frozen root tissue, except that the flow rate was 3.0 ml min^{-1} and a Zorbax C_{18} column (150 mm x 2.5 cm, Mississauga, ON, Canada) was used. The major peak was isolated, dried in a rotary evaporatory, and then the compound was dissolved in 510 μl of fresh methanol (Sigma). ^{1}H NMR, ^{1}H-^{1}H COSY, and HMBC spectra were measured on the isolated compound with a Bruker Advance 500 NMR spectrometer equipped with a Bruker 5 mm inverse tripe resonance TXI probe (Bruker Biospin, Rheinstetten, Germany) at the Saskatchewan Structural Science Centre, University of Saskatchewan. Chemical shifts (Table 1) (δ) were expressed in parts per million (ppm) and coupling constants (*J*) in Hertz (Hz).

To determine the configuration of the sugar moiety, the isolated compound was refluxed with 6 N HCl (5 ml) at 100°C for 2 h (Li et al., 2009b). The mixture was extracted with $CHCl_3$ to remove the aglycone, then the aqueous layer was neutralized with Na_2CO_3, filtered, and dried under vacuum. A portion of the aqueous residue was dissolved in H_2O for sugar analysis by thin layer chromatography (TLC) (Macherey-Nagel, SilG/UV254, 0.20 mm) with n-BuOH: HOAc:H_2O (6:1:2) as the solvent. TLC sample spots were detected by spraying aniline hydrogen phthalate reagent (100 ml n-butanol saturated by H_2O, 0.96 g aniline and 1.66 g phthalic acid) and heating. The absolute configuration of glucose was determined by chiral GC-FID analysis using an Agilent GC and a 0.25 mm×25 mm Hydrodexb-6-TBDM chiral capillary column (Macherey-Nagel, Germany). *β*-D-glucose (Sigma) was used as an authentic GC standard. A portion of the aqueous layer residue also was resuspended in 1 ml dichloromethane followed by the addition of 50 μl trifluoroacetic anhydride. The mixture was allowed to react at room temperature for 16 h and dried under a stream of nitrogen at room temperature. The sugar derivative was separated on the GC using the following temperature program: inlet temperature at 240°C; hydrogen carrier gas and a 1/20 split using nitrogen as the makeup gas; column temperatures starting at 120°C, ramping to 220°C at 50°C min^{-1} and maintenance for 12 min.

Microarray Total RNA was extracted from *Arabidopsis* seedlings grown on agar plates for 6 d with or without 4-MU supplementation using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA). cDNA was prepared using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Three biological replicates were collected for each sample. An *Arabidopsis* 26 K cDNA array (Qiagen, Valencia, CA, USA) was hybridized with Cy5- and Cy3-labelled probe pairs from the 4-MU treated and untreated samples. Probe labeling, hybridization, and washing were conducted using a CyScribe Post-Labeling kit according to the manufacture's instruction (Amersham Biosciences, Pittsburgh, PA, USA). Hybridized slides were scanned on a GenePix 4000B scanner (Axon Instruments Inc., Union City, CA, USA). The intensity (I) of each spot at λ_{546nm} and λ_{647nm} was transformed into a I_{546}:I_{647}ratio with Array-Pro Analyzer 4.0 (Media Cybernetics Inc., Bethesda, MD, USA). Microarray data initially were imported into BASE 1.2.16. The data then were filtered, normalized, and analyzed with Gene-Spring 6.1 (Silicon Genetics, Redwood City, CA, USA) as previously described (Zhao et al., 2009), and imported into MapMan for category analysis.

Real Time RT-PCR mRNA from the microarray experiment was converted to cDNA with SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative real time PCR analysis (qRT-PCR) was carried out using a Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA, USA) on an ABI PRISM StepOnePlus Real-Time PCR System (Applied Biosystems) as previously described (Gao et al., 2009; Li et al., 2011). The *Arabidopsis*

Table 1 ^{1}H-NMR data of 4-MU and 4-MU-Glc (500 MHZ) [a, b]

	4-MU	4-MU-*O*-*β*-*D*-Glc		4-MU	4-MU-*O*-*β*-*D*-Glc
2	–	–	10	–	–
3	6.10, *s*	6.10, *s*	-CH_3	2.43, *s*	2.36, *s*
4	–	–	1′	–	4.95, *d*, 7.6
5	7.59, *d*, 8.5	7.62, *d*, 8.5	2′	–	3.32–3.45, *m*
6	6.83, *dd*, 8.5, 1.7	7.04, *dd*, 8.5, 1.7	3′	–	3.32–3.45, *m*
7	–	–	4′	–	3.32–3.45, *m*
8	6.70, *d*, 1.7	6.97, *d*, 1.7	5′	–	3.64, *dd*, 11.8, 5.6
9	–	–	6′	–	3.82, *d*, 11.8

[a] Spectra were measured in MeOD, and chemical shifts are expressed in ppm

[b] Multiplicity and *J* values in Hz are given in parentheses

Ef-1α gene was chosen as an endogenous reference gene. Primer sequences for qRT-PCR were designed using Oligo Perfect Primer Design software provided by Invitrogen (http://tools.invitrogen.com/) (Table 2). For each pair of primers, gel electrophoresis and melting curve analyses were performed to ensure that only a single PCR amplicon of the expected length and melting temperature was generated. Each sample was assayed in triplicate, and data were analyzed using Step-One v2.0 (Applied Biosystems). The level of each mRNA was calculated using the mean threshold cycle (Ct) value and normalized to that of the reference gene *Ef-1α*. All results are shown as means of at least three biological replicates with corresponding standard deviations (SD).

Glucosyltransferase Activity Assay To generate constructs for GST-UGT expression, the full length open reading frames (ORFs) of *UGT73B4* and *UGT75B2* were amplified by PCR using primers 73B4-PET-F and 73B4-PET-R for *UGT73B4* and primers 75B2-PET-F and 75B2-PET-R for *UGT75B2* (Table 2), and cloned into the pET102/D/TOPO vector using the directional TOPO cloning system (Invitrogen, Carlsbad, CA, USA). All constructs were confirmed by DNA sequencing. The recombinant plasmids were transformed into *Escherichia coli* BL21 Star (DE3) cells (Invitrogen, Carlsbad, CA, USA), and the protein was purified as a GST fusion according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Each glucosyltransferase activity assay mix (2 ml) contained 100 μg of recombinant protein, 50 mM Tris-HCl, pH 7.0, 10 mM β-mercaptoethanol, 10 mM UDP-glucose, and 1 mM 4-MU as substrate. The reaction mix was incubated at 28°C for 1 h, stopped with the addition of 200 μM trichloroacetic acid, and subjected to reverse-phase HPLC analysis as described above. Absorbance at λ_{320} was used to monitor chromatogram profiles, and the identity of peaks (compounds) was confirmed by comparing retention times and photo-diode array profiles with authentic standards.

Statistical Analysis All experiments were conducted in triplicate and mean data (±SD) analyzed with ANOVA for significance (LSD at $P<0.05$) using SAS 9.0 (SAS Institute, Inc. Cary, NC, USA), unless otherwise indicated. For microarray analysis, log ratios of intensity after

Table 2 Primers used in this study

	Gene	AGI code	Sequences
GAUT2-F	*GAUT2*	AT2G46480	CCTGCCTTTACCGATGAGAA
GAUT2-R			AAGGTGGAAAACATGCCTTG
UGT73B4-F	*UGT73B4*	AT2G15490	GGAGAAGATCGGTGTTCCAA
UGT73B4-R			CAGGGAGACCAGGGATTACA
UGT73C1-F	*UGT73C1*	AT2G36750	CGCACATAATGCACCAAAAC
UGT73C1-R			CCGTCAAGGAAGTCTTTCCA
UGT75B2-F	*UGT75B2*	AT1G05530	GACACCGAGCCATAGGTTGT
UGT75B2-R			CCCTCACACCTGTCTTCCAT
PIN2-F	*PIN2*	AT5G57090	CCATGATAACGCCGCGAGCTTC
PIN2-R			TGACGAGGGCTTGGAGCTTTGC
PIN3-F	*PIN3*	AT1G70940	CCATCTTCGCCGTCCCTCTCCT
PIN3-R			CGAGGCTACCGGAGCGAGTGAA
IAA14-F	*IAA14*	AT4G14550	AAAGTCCGGCCAAGTCGGGTGT
IAA14-R			CCTCTGCTTCGCCGCTCTTCTG
GH3-F	*GH3*	AT2G14960	CGTTGCTGCCTTGGGATGGAAG
GH3-R			GGGCTAAAATGCGCCGACACAA
YUCCA1-F	*YUCCA1*	AT4G32540	ATCGGAGCTGGCCCTTCTGGTC
YUCCA1-R	*TIR1*	AT3G62980	CCAGGAGGGGTAACCGGCAAAA
TIR1-F			ATCGGGAACTGCTACGCCGTGA
TIR1-R			TCCAAGCAATCGTCGGTGACCA
EF-1α-F	*EF-1α*	AT1G07920	GCAAGGGCCTCAGCTCTG
EF-1α-R			ACAAGCCGTCTGGGTATATGTTAGC
73B4-PET-F	*73B4-PET*	AT2G15490	CGCTCGAGATGAACAGAGAGCAAATTCATA
73B4-PET-R			CGGAATTCCTACTTTCTACCATTCAGCTCTTC
75B2-PET-F	*75B2-PET*	AT1G05530	CGCTCGAGATGGCGCAACCGCATTTT
75B2-PET-R			CGGAATTCTCAAAACAGACTCTTCACAA

background correction were scaled to have similar distribution across and consistency among arrays. The ANOVA model "log $(y_{ijkgr})=\mu+A_i+D_j+T_k+G_g+AG_{ig}+DG_{jg}+TG_{kg}+\varepsilon_{ijkr}$" was employed to detect differentially expressed genes using the normalized data, where log (y_{ijkgr}) represents the background-corrected and normalized natural logarithm of the intensity of the r^{th} replicate of gene g on array i, with dye j, and treatment/condition k; μ represents the average natural logarithm of gene intensity over all the genes, arrays and dyes; A, D, T, and G represent the array, dye, treatment and main gene effect, respectively; and ε_{ijkr} represents normal distribution with mean zero (Li et al., 2009a).

Results

Identification of 4-MU as a Root Growth Effector A set of 128 natural compounds was screened for their impact on *Arabidopsis* seed germination, primary root growth, root architecture, and seedling establishment. Out of this screening, seven coumarins and five phenolic acids delayed *Arabidopsis* seed germination and inhibited root growth. One coumarin and two ginsenosides inhibited root growth alone, and one phenolic acid and one flavonoid inhibited seed germination alone (Supplementary Table 1). Of all compounds tested, 4-MU affected all three parameters, including root architecture. Since 4-MU was the only compound to affect root architecture, it became the focus of the remainder of this study

Seed germination was inhibited by a range of 4-MU treatments. Germination rates were 90% and 75% with 125 μM and 250 μM 4-MU, respectively. Seeds were unable to germinate when the concentration of 4-MU was increased to 500 μM (data not shown). Primary root growth was inhibited in 6-d-old seedlings grown on medium supplemented with 125 μM or 250 μM 4-MU (Fig. 1a,b,c). Primary root length was reduced by 25% and 75%, respectively, compared to untreated control seedlings over a 22 d time period (Fig. 2a), but growth of cotyledons, hypocotyls and rosette leaves was not affected at these concentrations (Figs. 1a,b, c and 2b). Accompanying the negative effect on primary root growth, the development of root hairs on primary roots also was affected by 4-MU. Approximately 20% of root hairs on primary roots were bulbous at 125 μM 4-MU (Fig. 1h), while very few root hairs were fully emerged at 250 μM 4-MU (Fig. 1i). After exposure to 125 μM 4-MU, lateral roots began to form as buds by day 14, and abundant lateral roots had formed by 22 days, even though primary root growth was hindered (Fig. 2c). Since accumulation of coumarins can be detected in plant tissues under UV light (Chong et al., 2002; Kai et al., 2008), the 6-d-old *Arabidopsis* seedlings grown on media supplemented with 4-MU were compared with untreated roots under UV light. Consistent with the

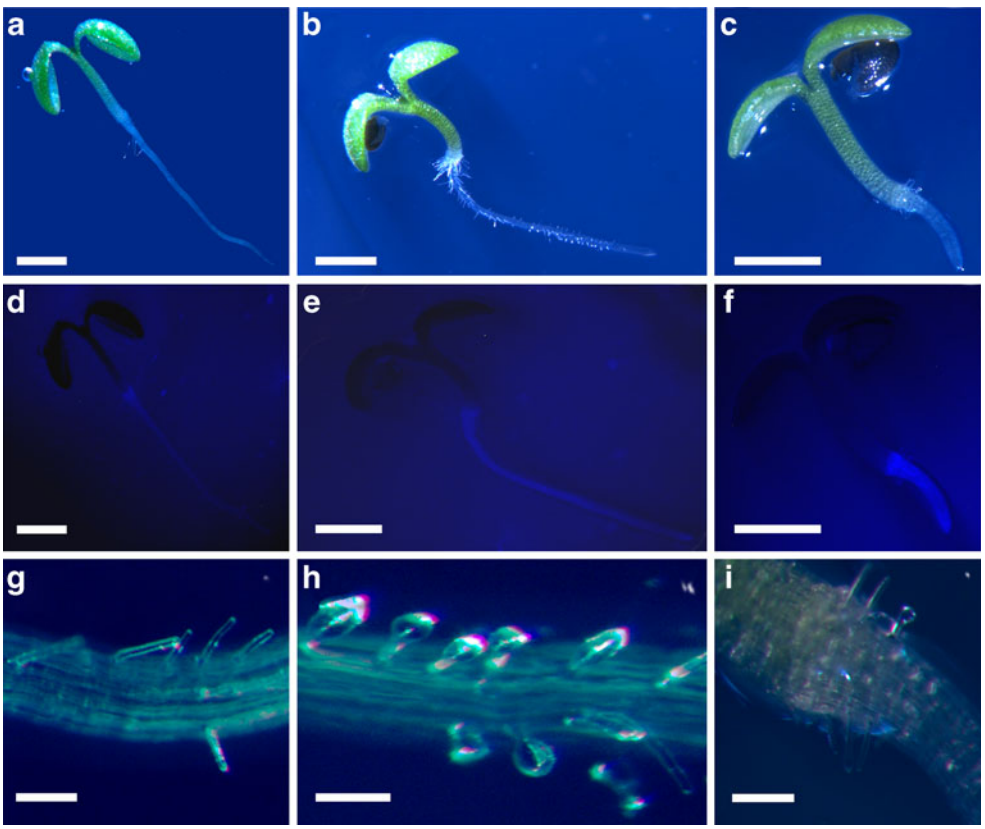


Fig. 1 The effect of exogenous 4-MU on growth of primary roots. *Arabidopsis* seedlings were grown vertically on media supplemented with different concentrations of 4-MU for 6 d. **a** An untreated seedling. **b** A seedling treated with 125 μM 4-MU. **c** A seedling treated with 250 μM 4-MU. (**d**, **e** and **f**) UV (325 nm) channel of (**a**), (**b**) and (**c**) showing the accumulation of fluorescent 4-MU in primary roots. (**g**, **h** and **i**) Root hairs of (**a**), (**b**) and (**c**) showing bulbous root hairs on the primary root after treatment with 125 μM 4-MU, and inhibition of root hair formation after treatment with 250 μM 4-MU. Scale bars in **a**–**f**, 1 cm; in **g**–**i**, 2 mm

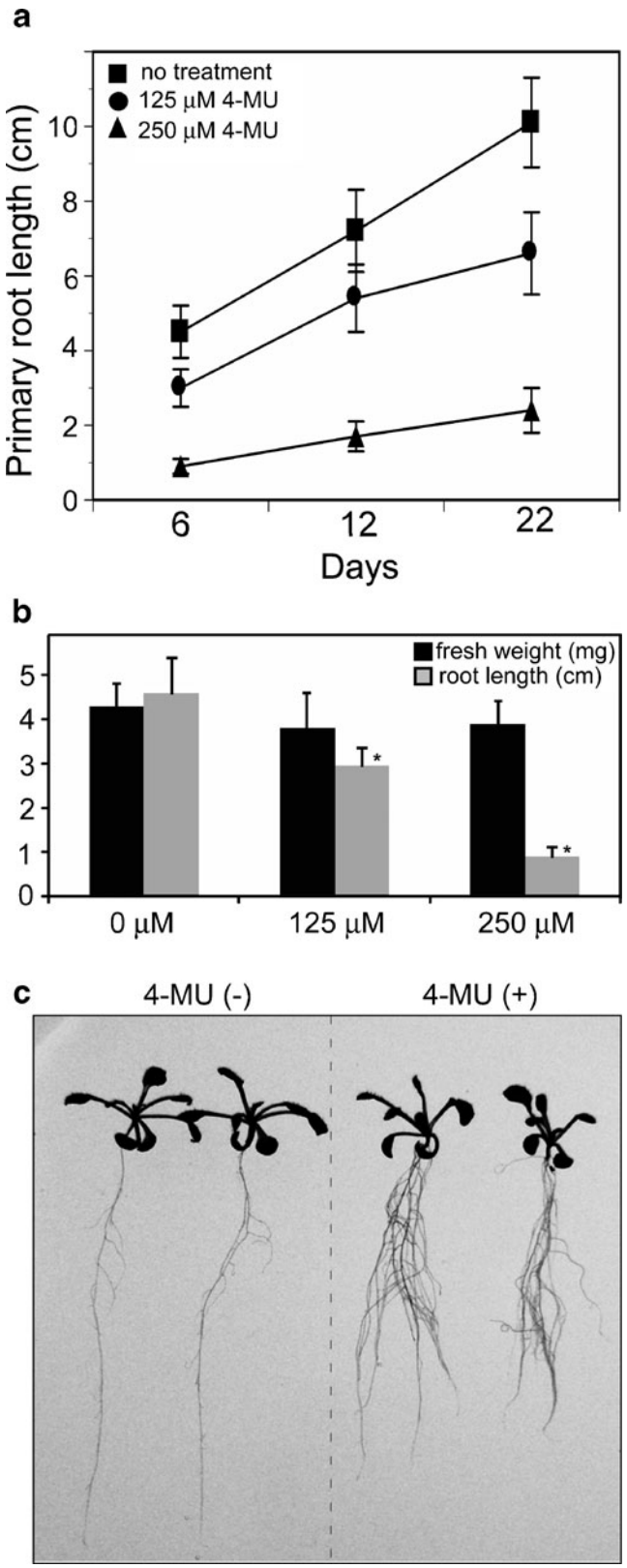


Fig. 2 The effect of 4-MU on *Arabidopsis* growth. **a** Time course of the effect of 4-MU on primary root elongation over 22 d. Values represent the means±SD of three replicates. **b** Fresh weight of aerial parts of plants (mg FW per 10 seedlings without roots) and root length (cm) of 6-d-old plants in the presence or absence of 4-MU. Data represent the mean±SD from 6 independent experiments. Asterisks indicate significant difference between treated and untreated plants based on a student test ($P<0.05$). **c** Root system architecture and aerial vegetation of 22-d-old plants showing root branching in the presence (right) of 125 µM 4-MU, very little branching without 4-MU (left), and normal rosette growth under both conditions

inhibition of *Arabidopsis* root growth by 4-MU, blue fluorescence increased in roots of seedlings treated with this compound (shown for 250 µM 4-MU in Fig. 1e and f).

Accumulation and Glucosylation of 4-MU in Arabidopsis Roots To confirm the presence of 4-MU in *Arabidopsis* roots, we analyzed the phytochemical composition of roots of 6-d-old seedlings by HPLC-UV/MS. In MeOH extracts from roots exposed to 125 µM 4-MU, 4-MU and a large additional peak were detected at *Rt* 36.5 min and *Rt* 23.0 min, respectively. Neither of these peaks was detected in the roots of untreated seedlings (Fig. 3a). Comparison of the ^{1}H NMR spectrum of the new reaction product with that of 4-MU (Supplementary Data Figs. 2 and 3) showed no significant chemical shifts in the aromatic proton signals, but an additional set of sugar moiety signals appeared at δ 4.95 (1H, *d*, J=7.6 Hz), 3.82 (1H, *d*, J=11.8 Hz), 3.62 (1H, *dd*, J=5.6, 11.8 Hz), and 3.32-3.45 (3H, m) (Table 1). Combinational ^{1}H-^{1}H COSY and HMBC NMR spectra (Supplementary Data Figs. 4 and 5) were used to elucidate the structure. The sugar moiety was identified as glucose on the basis of an acid hydrolysis reaction, which gave a reaction product with the same R_f on a TLC plate as an authentic glucose standard compound (data not shown). The coupling constant $J_{H1', H2'}$ (7.6 Hz) of the new enzyme reaction product, followed by chiral GC analysis, indicated an absolute configuration of β-D-glucose. The new reaction product was defined as $C_{16}H_{18}O_8$ [4-MU-β-D-glucopyranoside (4-MU-Glc)] based on its HRESIMS ($[M+Na]^+$, *m/z* 361.0906, calc. 361.0899) (Fig. 3b). This indicates that 4-MU is partially transformed into 4-MU-Glc by glucosylation after uptake in *Arabidopsis* roots.

To gain additional insight into how *Arabidopsis* plants react to 4-MU by glucosylation after seed germination, we conducted a microarray analysis of 6-d-old seedlings grown on medium containing 125 µm 4-MU (Supplementary Data Tables 2 and 3). A total of 316 genes showed statistically significant up-regulation, while 243 showed statistically significant down-regulation. Genes determined to be up-regulated or down-regulated ≥ 2-fold in the treated relative to the untreated sample were catalogued and placed into twelve categories based on gene function according to *Arabidopsis* Gene Ontology (Supplementary Data Fig. 6). Although differences between treated and untreated samples were noted for individual genes, the number of up-regulated and down-regulated genes were similar in the categories of response to stress (51 vs. 48), response to

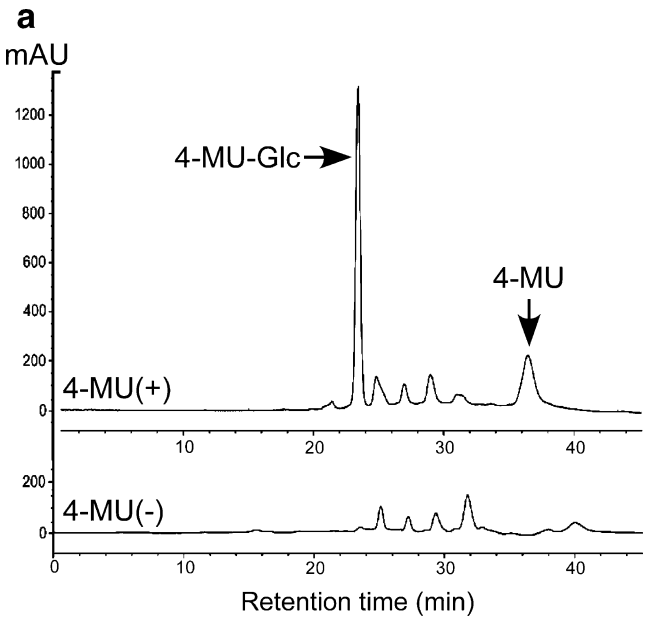


Fig. 3 Glucosylation of 4-MU in *Arabidopsis* roots. **a** HPLC-UV profiles (λ_{325}) of 80% methanol extracts from roots of 6-d-old seedlings treated with (upper panel) or without (bottom panel) 125 μM 4-MU. A new product was identified as 4-MU-*β*-D-glucoside (4-MU-Glc) in the 4-MU treated root extracts. **b** Schematic of the UGT-catalyzed biotransformation of 4-MU to 4-MU-Glc in *Arabidopsis* roots

abiotic and biotic stresses (49 vs. 44), and transportation (32 vs. 25), respectively. It was notable that most of the genes with altered expression did not have known functions (99 vs. 145, respectively). Four genes encoding uridine diphosphate (UDP) glycosyltransferases (UGTs) were up-regulated more than two-fold in the 4-MU treated seedlings, these included *UGT73B4*, *UGT73C1*, *UGT75B2*, and *GAUT2* (At2g46480). Consistent with the microarray data, qRT-PCR analysis revealed significant up-regulation of these four *UGT* genes in roots of 6-d-old seedlings exposed to 125 μm 4-MU (Fig. 4). Since UGT73B4, UGT73C1, and UGT75B2 are active on hydroxycoumarins, scopoletin, and esculetin (Lim et al., 2003), we tested whether 4-MU could be glucosylated by these UGTs using purified recombinant UGT73B4 and UGT75B2 enzymes. Reverse-phase HPLC

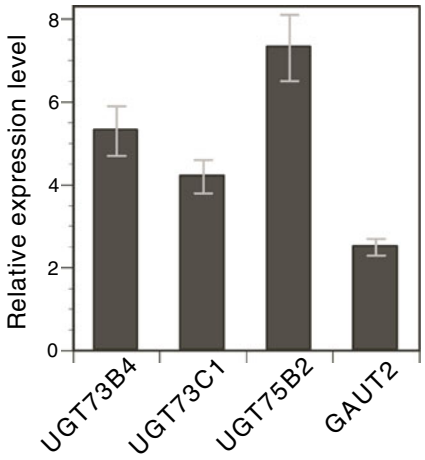


Fig. 4 Induction of glycosyltransferase genes by 4-MU in *Arabidopsis* roots. Quantitative RT-PCR analysis was used to analyze expression of genes encoding glycosyltransferases in the roots of 6-day-old seedlings treated with 125 μM 4-MU relative to levels from untreated seedlings. *Ef-1α* was used as a reference gene

and the identification of compounds in the post-reaction mixture showed a product with a retention time (22.1 min) and a photo-diode array profile identical to the authentic standard 4-MU-Glc in the reaction with UGT73B4, indicating that 4-MU is a substrate of this UGT (Fig. 5). UGT75B2 enzyme activity was not detected with 4-MU (data not shown).

Involvement of Auxin Efflux in 4-MU-Induced Lateral Root Formation Auxin plays a key role in controlling lateral root

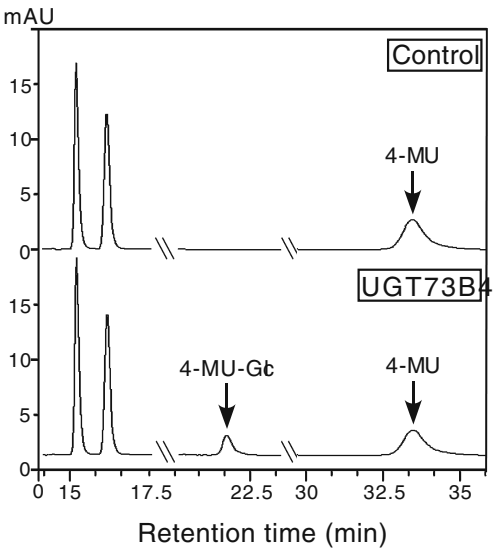


Fig. 5 HPLC-UV profiles of the reaction products of UGT73B4 recombinant protein incubated with 4-MU. Reaction products of GST protein (Control) or GST-UGT73B4 fusion (UGT73B4) were subjected to HPLC analysis and the products were detected at λ_{320}, (RT, 22.1 min) and confirmed by NMR, showing that UGT73B4 catalyzes the conversion of 4-MU to a 4-MU-Glc

formation (Fukaki and Tasaka, 2009). To test whether lateral root formation induced by 4-MU treatment was accompanied by an alteration in auxin signaling, we performed qRT-PCR analysis on genes involved in either auxin signal transduction, flux, or biosynthesis using total RNA extracted from 4-MU-treated roots of 14-d-old seedlings when lateral root branching has been initiated and *DR5::GUS* was actively expressed (Supplementary Data Fig. 1). For auxin flux facilitator genes, we chose *PIN2* and *PIN3* in preference to the other five *PIN* genes, which have additional or other functions. For example, *PIN1* is involved in vascular development, phyllotaxis, vein formation, embryogenesis, and lateral organ development (Petrásek and Friml 2009). *PIN2* and *PIN3* were up-regulated by 4-MU, whereas the expression of an auxin receptor *TIR1*, a rate-limiting for the auxin biosynthetic gene *YUCCA1* (Zhao et al., 2001), and an auxin-responsive GH3 family member At2g14960 (Mishra et al., 2009) was unchanged relative to untreated seedlings (Fig. 6a). Additionally, the transcript level of *IAA14/SLR1*, one of the most important auxin responsive AUX/IAA genes involved in lateral root initiation (Fukaki et al., 2002; Fukaki and Tasaka, 2009), was elevated by 4-MU. This analysis suggested that 4-MU affected auxin redistribution rather than biosynthesis. To eliminate the possibility that 4-MU treatment affects endogenous auxin biosynthesis, β-glucuronidase (GUS) activity under the control of the synthetic auxin-responsive promoter *DR5* (DR5::GUS) was determined in roots of 14-d-old seedlings grown on medium supplemented with 125 μM 4-MU. MUG activity (as measured by a fluorometric assay) in *DR5::GUS*—transformed *Arabidopsis* lines grown in the presence of 4-MU showed no difference compared to lines without 4-MU treatment (Fig. 6b), indicating that 4-MU had no effect on the regulation of auxin biosynthetic genes.

Alteration of Actin Filament Organization by 4-MU Treatment The actin cytoskeleton is important for root growth and is also thought to play a role in the polarization of auxin fluxes (Dhonukshe et al., 2008; Nick et al., 2009). We, therefore, examined the organization of the actin cytoskeleton system in roots in response to 4-MU. *Arabidopsis* plants were transformed with the actin-binding domain (ABD)-GFP protein fusion construct, which specifically binds filamentous (F)-actin (Motes et al., 2005) and allows visualization of the F-actin cytoskeleton. ABD-GFP florescence strongly accumulated in the root caps of untreated 6-d-old seedlings (Fig. 7b), whereas GFP fluorescence was observed in the meristematic tissues of seedlings treated with 125 μM 4-MU (Fig. 7e). Alteration of the root F-actin organization in plants treated with 125 μM 4-MU was accompanied by an abnormal root tip phenotype in which root caps appeared to be physically detached with irregularly shaped cells (Fig. 7d,f).

Discussion

We have characterized the response of the *Arabidopsis* root system to treatment with 4-MU. In the presence of this compound, seed germination was inhibited, primary root growth and root hairs were reduced, and lateral root emergence was stimulated, while cotyledons and seedling rosette leaves were unaffected (at least up to 22 days of evaluation). At high concentrations (500 μM), germination

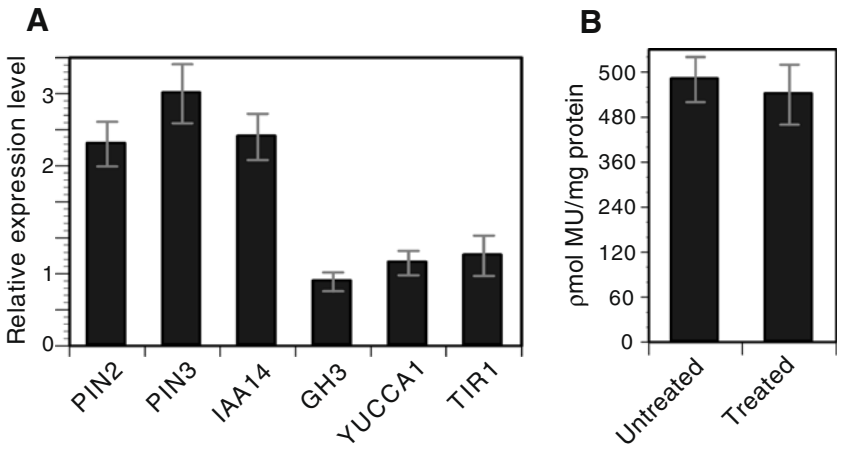


Fig. 6 (A) Transcript abundance of auxin biosynthesis, efflux, signalling, and responsive genes after 4-MU treatment. Expression was tested for the auxin efflux facilitators *PIN2* and *PIN3*, the auxin-responsive genes *IAA14* and *GH3* (At2g14960), the major auxin biosynthetic gene *YUCCA1* and the auxin receptor *TIR1*. Real time PCR was used to examine transcript levels of the six genes are relative to that in untreated *Arabidopsis* seedlings. *Ef-1α* was used as a reference gene. (B) Auxin biosynthesis as detected by GUS activity levels in *DR5::GUS* transgenic seedlings untreated or treated 125 μM 4-MU. Values are the means ± SD of triplicate samples

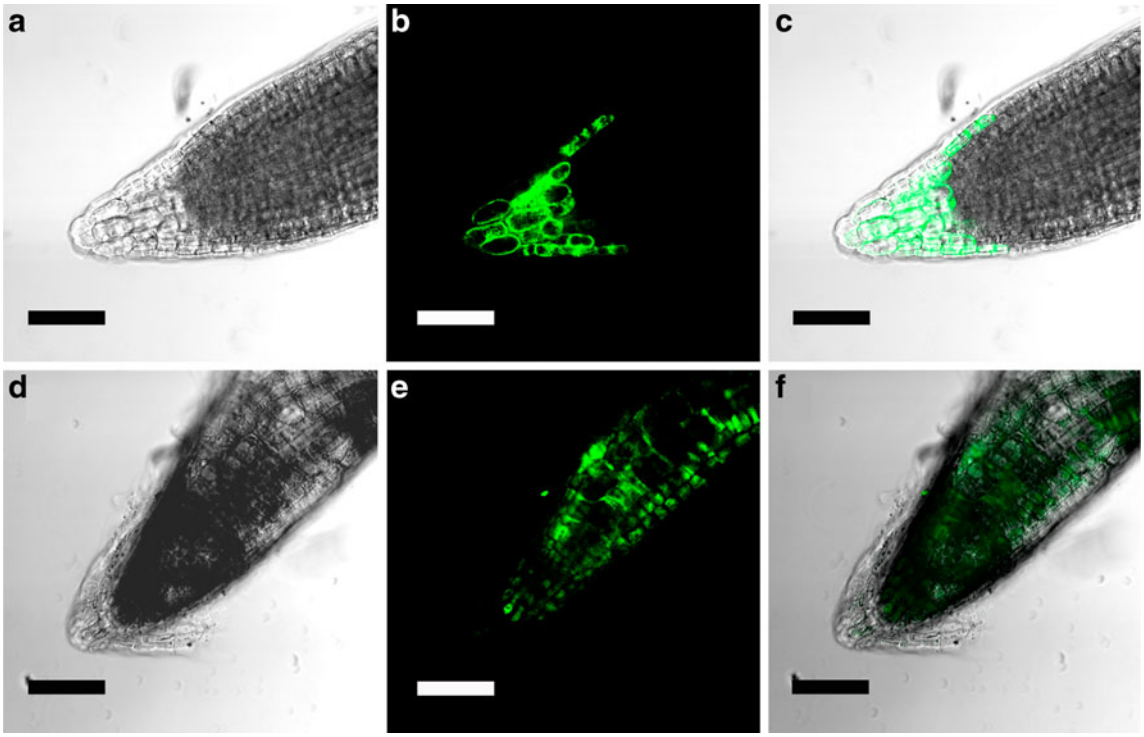


Fig. 7 Alteration of actin cytoskeleton organization in *Arabidopsis* root tips in response to 4-MU. Primary root tips of 6-d-old *35S::ABD-GFP* transformed seedlings were examined by fluorescence microscopy. (**a**, **b** and **c**) A root tip in the absence of 4-MU. (**d**, **e** and **f**) A root tip treated with 125 μM 4-MU. Brightfield (**a** and **d**), GFP channel (**b** and **e**), and merged (**c** and **f**) images are shown. Scale bar, 100 μm

was completely inhibited. Transcripts of three UGTs (UGT73B4, UGT73C1, UGT75B2) also were up-regulated after treatment with 4-MU, and a recombinant UGT73B4 enzyme could catalyze the glucosylation of 4-MU *in vitro* into the same end product (4-MU-Glc) that was accumulated with 4-MU *in vivo* in roots of the treated plants.

Plants have evolved complex pathways for responding to chemicals and toxins, such as glutathione sulphotransferase (GST)-catalyzed conjugation of glutathione and UGT-catalyzed glucosylation of diverse acceptors (Edwards et al., 2000; Gandia-Herrero et al., 2008). Glucosylation alters the properties of acceptor chemicals, such as their bioactivity, stability, hydrophilicity, and subcellular localization (Bowles et al., 2005). UGTs are involved in the synthesis and modification of glucoconjugates via transfer of sugars to a wide range of acceptor molecules that include plant secondary metabolites, phytotoxins, and xenobiotics (Bowles et al., 2005). UGT73B4 and several other UGTs have been shown to glucosylate coumarins, scopoletin, and esculetin, the flavonoid quercetin and 2,4,6-trinitrotoluene (TNT) (Lim et al., 2003; Gandia-Herrero et al., 2008). Hence, UGT-catalyzed glucosylation of 4-MU may represent a mechanism to inactivate this compound in *Arabidopsis* roots.

Auxin and its polar transport and redistribution are key factors controlling lateral root initiation, formation, and development (Nibau et al., 2008; Fukaki and Tasaka, 2009; Peret et al., 2009; Vanneste and Friml, 2009). Lateral root emergence was correlated with changes in auxin responsive gene expression in *Arabidopsis* challenged with 4-MU. Genes that encode the auxin efflux facilitators PIN2 and PIN3 were strongly up-regulated by 4-MU, and expression of the auxin receptor TIR1 or the key auxin biosynthetic enzyme YUCCA1 was unchanged in the presence of 4-MU. The F-actin fibrils in the primary root also were disrupted, and the root cap was detached under 4-MU exposure. These observations suggest that 4-MU induces lateral root emergence and root branching through auxin redistribution rather than auxin biosynthesis and does so in concert with (and as a consequence of) the inhibition of primary root growth.

Small compounds with impacts on cellular activity are useful, potent chemical tools for defining protein function (Walsh and Chang, 2006) and have provided insight into regulatory mechanisms, e.g., toyocamycin inhibition of auxin signaling (Hayashi et al., 2009). Our identification and characterization of 4-MU as a positive regulator of *Arabidopsis* root branching as a consequence of primary root inhibition is a starting point for continued chemical

genetics investigations aimed at identifying the cellular target of 4-MU. At this point, we also anticipate that 4-MU will prove to be a useful chemical tool for studying root biology and development in crop plants, particularly the *Brassicas* which are genetically close to *Arabidopsis*.

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Although cover crops provide added value to commercial crops in agronomic systems, many species used as coverage are toxic due to the high concentrations of phytochemicals. Velvetbean (*Mucuna pruriens*. DC. var. utilis) is an example of an efficient cover crop with several highly active natural products. It has been grown widely in tropical areas as a soil-improving crop, a cover crop to control weeds, green manure, and a forage plant (Anaya, 1999). Many secondary compounds are produced by velvetbean, but the major one is the non-protein amino acid, L-3,4-dihydroxyphenylalanine (L-DOPA), which represents from 0.5 to approximately 1.5% of the fresh weight of leaves (Fujii et al., 1991) and from 6 to 9% of the seeds (Rehr et al., 1973). L-DOPA is released from the roots of velvetbean (Furubayashi et al., 2005), and the soil concentration can reach 50 mg l^{-1} in the vicinity of the roots (Nishihara et al., 2005). This concentration is sufficient to affect the growth of neighboring plants due to both reduced seed germination and suppression of root growth (Fujii, 1999).

The allelopathic action of L-DOPA has been attributed to its ability to reduce the growth of several plant species, including weed species such as wild mustard (*Sinapis arvensis*), creeping thistle (*Cirsium arvense*), field poppy (*Papaver rhoeas*), henbit (*Lamium amplexicaule*), and crop species such as wheat (*Triticum vulgare*), barley (*Hordeum vulgare*), pumpkin (*Cucurbita moschata*), lettuce (*Lactuca sativa*), cucumber (*Cucumis sativus*), and cabbage (*Brassica oleraceae*) (Nishihara et al., 2004; Topal and Kocaçalişkan, 2006). In previous work (Soares et al., 2007) we observed that the reduction of soybean (*Glycine max*) root growth by L-DOPA is associated with increases in the enzyme activities of the phenylpropanoid pathway, such as phenylalanine ammonia-lyase (PAL) and peroxidase (POD), and increases in phenolic compounds and lignin content. Based on these findings, we hypothesized that one mode of action of L-DOPA may be related to its effects on the phenylpropanoid pathway, which triggers excessive production of lignin and a reduction in soybean root growth.

Hachinohe and Matsumoto (2007a) hypothesized that the phytotoxicity of L-DOPA might be due to reactive oxygen species (ROS) formed during its oxidation to melanin. Using suspension-cultured carrot (*Daucus carota*) cells, they observed that exogenous L-DOPA was metabolized to melanin, and ROS species generated by this process caused oxidative damage followed by growth reduction. It is known that melanin biosynthesis from L-DOPA requires the action of the polyphenol oxidase (PPO) and POD enzymes (Albrecht and Kohlenbach, 1990; Nishihara et al., 2004) in an oxidative process that can generate ROS, such as H_2O_2, $^1O_2$, $O_2^{\bullet -}$, and $OH^{\bullet}$ (Riley, 1997). The oxidative damage induced by ROS includes chlorophyll degradation, structural and functional changes of proteins, DNA fragmentation, ion leakage, lipid peroxidation, and cell death (Moller et al., 2007). However, there also is evidence that L-DOPA can act as an antioxidant agent (Spencer et al., 1996). Randhir et al. (2006) suggested that L-DOPA is an effective direct quencher of free radicals and a potential modulator of the antioxidant enzyme response. Also, Gülçin (2007) proposed that L-DOPA has an antioxidant property similar to synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol and Trolox.

In view of this apparent contradiction concerning the oxidative or antioxidative property of L-DOPA, the purpose of this study was to investigate further the mode of action of L-DOPA in soybean roots by evaluating its effects on several parameters related to the oxidative stress condition. The production of $O_2^{\bullet -}$ and H_2O_2 and the level of lipid peroxidation as well as the activities of some enzymes of the antioxidant defense systems, including PPO, POD, superoxide dismutase (SOD), and catalase (CAT) were measured. The production of melanin from L-DOPA also was evaluated.

Methods and Materials

General Procedures Soybean (*Glycine max* L. Merr. cv. BRS-184) seeds were surface-sterilized with 2% sodium hypochlorite for 5 min, rinsed extensively with deionized water, and dark-germinated (at 25°C) on two sheets of moistened filter paper. Twenty-five 3-d-old seedlings of uniform size were supported by an adjustable acrylic plate and dipped into a glass container (10×16 cm) filled with 200 ml of half-strength Hoagland's solution (pH 6.0), with or without 0.1 to 1.0 mM L-DOPA. All nutrient solutions were buffered with 17 mM potassium phosphate buffer. The containers were kept in a growth chamber (at 25°C, with a 12:12 hr L:D photoperiod and a photon flux density of 280 μmol m^{-2} s^{-1}) for 24 hr. L-DOPA was purchased from Sigma-Aldrich (St Louis, MO, USA), and all other reagents used were of the purest grade available.

Quantification of Melanin Fresh roots (50 mg) were incubated with 1 ml of 1 N NaOH at 100°C. After 1 hr, the absorbance was recorded at 470 nm. Melanin content was calculated by using a standard curve prepared with known concentrations of melanin (Abdel-Naser et al., 2003). The results are expressed as μg melanin g^{-1} fresh weight.

Enzyme Assays PPO activity was estimated according to the method of Oktay et al. (1995). Fresh roots (0.5 g) were ground in a mortar with 0.01 g of polyvinylpolypyrrolidone (PVPP) and 5 ml of 0.05 M phosphate buffer (pH 7.0). The

extract was centrifuged (2,200×*g*, 5 min, 4°C), and the supernatant was used to determine the activity of PPO. The reaction mixture contained 3.6 ml of 0.05 M phosphate buffer (pH 6.0), 0.1 ml of 0.1 M catechol, and 1 ml of enzyme extract. The mixture was vortexed for 15 sec, followed by incubation at 30°C for 30 min; after transfer to an ice bath, 0.2 ml of 1.4% perchloric acid were added, and the mixture was vortexed. After 10 min, the absorbance of the solution was followed for 5 min at 395 nm. One unit of PPO activity was defined as an increase in the A_{395} by 0.001 min^{-1} ml^{-1}. The enzyme activity is defined as unit g^{-1} fresh weight.

SOD activity was assayed by using the photochemical nitroblue tetrazolium (NBT) method (Giannopolitis and Ries, 1977). Fresh roots (0.5 g) were ground in a mortar with 0.01 g of PVPP and 2 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1.0 mM EDTA. After centrifugation (2,200×*g*, 20 min, 4°C), the supernatant was used for the determination of the enzyme activity. The reaction mixture (1.5 ml) contained 75 μM NBT, 4 μM riboflavin, 13 μM methionine, 50 mM phosphate buffer (pH 7.8) containing 1.0 mM EDTA, and 20 μl of enzyme extract. To exclude eventual interference on SOD activity, parallel controls with L-DOPA or melanin added in the reaction mixture without enzyme preparation were undertaken under the same experimental conditions. The reaction was initiated by placing the reaction-mixture tubes under 15-W fluorescent lamps (56 μmol m^{-2} s^{-1}) for 10 min. The reaction was stopped by keeping the tubes in the dark for 10 min. The photoreduction of NBT (formation of purple formazan) was measured at 560 nm. One unit of SOD enzyme activity was defined as the amount of enzyme required to produce a 50% inhibition of the reduction of NBT. SOD activity is expressed as unit g^{-1} fresh weight.

For the POD assay, fresh roots (0.5 g) were ground in a mortar with 0.01 g of PVPP and 5 ml of 67 mM phosphate buffer (pH 7.0). The extract was centrifuged (2,200×*g*, 5 min, 4°C), and the supernatant was used to determine the activity of POD. The enzyme activity was determined according to the method of Cakmak and Horst (1991), with modifications. The reaction mixture (3 ml) contained 25 mM sodium phosphate buffer (pH 6.8), 2.58 mM guaiacol, and 10 mM H_2O_2. The reaction was initiated by the addition of the enzyme extract. The oxidation of guaiacol was followed for 5 min at 470 nm, and the enzyme activity was calculated from the extinction coefficient (25.5 mM^{-1} cm^{-1}). POD activity is expressed as μmol tetraguaiacol min^{-1} g^{-1} fresh weight.

For the CAT assay, fresh roots (1.0 g) were ground in a mortar with 0.01 g of PVPP and 1 ml of 0.1 M potassium phosphate buffer (pH 7.0). The extract was centrifuged (2,200×*g*, 10 min, 4°C), and the supernatant was used to determine the activity of CAT. The enzyme activity was determined according to method of Tománkóva et al. (2006). The reaction mixture (0.5 ml) contained 60 mM H_2O_2 in 60 mM sodium phosphate buffer (pH 7.4). The reaction was initiated by the addition of 0.1 ml of enzyme extract. The mixture was incubated for 4 min at 37°C, and the reaction was stopped by the addition of 0.5 ml of 32.4 mM ammonium molybdate. The yellow complex of molybdate and H_2O_2 was measured at 405 nm, and the enzyme activity was calculated from the extinction coefficient (0.0655 mM^{-1} cm^{-1}). CAT activity is expressed as μmol H_2O_2 consumed min^{-1} g^{-1} fresh weight.

Reactive Oxygen Species (ROS) Content Superoxide anion $\left(O_2^{\bullet -}\right)$ was determined colorimetrically, according to the method of Wu and von Tiedemann (2002). Roots (0.5 g) were homogenized with 2.7 ml of 50 mM phosphate buffer (pH 7.8). The homogenate (900 μl) was incubated with 100 μl of hydroxylamine (15 min, 30°C), and then 350 μl of the mixture were mixed with 350 μl of 17 mM sulfanilamide (in 30% acetic acid) and 350 μl of 7 mM naphthalene. Absorbance was measured at 540 nm. Results are expressed as μmol $O_2^{\bullet -}$ g^{-1} fresh weight.

The H_2O_2 content was determined colorimetrically, according to the method of Hsu and Kao (2007), with modifications. Roots (2 g) were homogenized in 3 ml of 50 mM phosphate buffer (pH 6.8). The homogenate was centrifuged (2,200×*g*, 20 min), and then 3 ml of the extracted solution were mixed with 1 ml of 0.1% titanium chloride in 20% (v/v) H_2SO_4. The mixture was centrifuged at 2,200×*g* for 15 min. Absorbance of the supernatant was measured at 410 nm by using the reaction mixture without the tissue extract as a blank. The H_2O_2 content was calculated from the extinction coefficient (0.25 mM^{-1} cm^{-1}). Results are expressed as μmol H_2O_2 g^{-1} fresh weight.

Lipid Peroxidation After 24 hr of incubation, seedlings were removed, and the roots were detached for the determination of the level of lipid peroxidation in terms of malondialdehyde (MDA). Fresh roots (0.25 g) were extracted with 2.5 ml of 67 mM phosphate buffer (pH 7.0). The extract was centrifuged (10,000×*g*, 15 min, 4°C), and the supernatant was used to determine the content of MDA (Doblinski et al., 2003). An aliquot of 0.5 ml of the supernatant was added to 4 ml of 0.5% of thiobarbituric acid (prepared in 20% of trichloroacetic acid). The mixture was heated in boiling water for 10 min and then quickly cooled in an ice-bath; samples were then centrifuged (3,500×*g*, 5 min). Absorbance of the supernatant was recorded at 532 nm, and this value was subtracted from the nonspecific absorbance (at 600 nm) value. The quantity of MDA was calculated from the extinction coefficient (155 mM^{-1} cm^{-1}). The level of lipid peroxidation is expressed as nmol MDA g^{-1} fresh weight.

Statistical Analysis The experimental design was completely randomized, and each plot was represented by one glass container with 25 seedlings. The data are expressed as the mean of three independent experiments ± SE. Data were analyzed with Mintab® package (Version 15, Minitab Inc., USA), and tested for normality (Kolmogorovy Smirnov-test) and homogeneity of variances (Levene's test) followed by one-way ANOVA. The differences between the parameters were evaluated by means of the Dunnett's multiple comparison test, and P values ≤0.05 were considered as statistically significant.

Results

Melanin Production from L-DOPA To verify whether melanin was formed from L-DOPA, soybean seedlings were grown in a nutrient solution with this allelochemical (0.1 to 1.0 mM). The L-DOPA increased the PPO activities by approximately 30%, regardless of its concentration, when compared with the control (Fig. 1a). In addition, Fig. 1b reveals that the roots exposed to L-DOPA produced a significant amount of melanin. After treatment with 0.1 to 1.0 mM L-DOPA, the melanin content reached from 0.44 to 1.23 μg g^{-1} fresh weight, respectively.

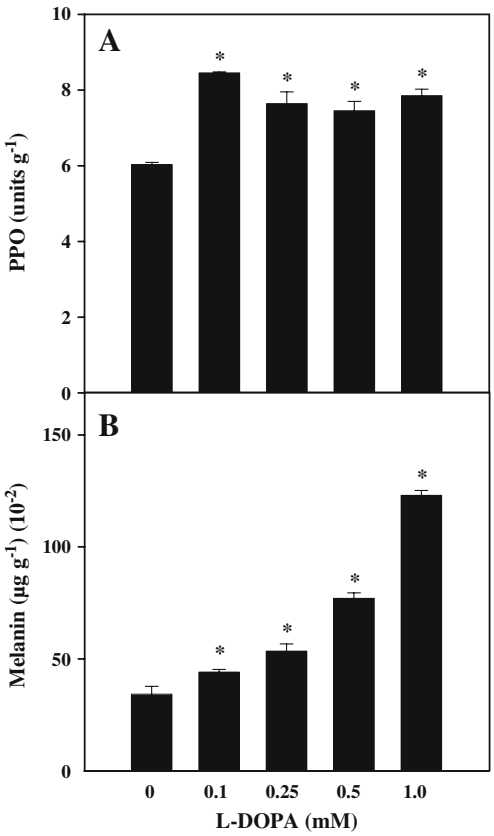


Fig. 1 The effects of different concentrations of L-DOPA on the activity of polyphenol oxidase (PPO) and the melanin contents in soybean roots. *Values (N=3±SE) differ statistically (Dunnett's multiple comparison test) from the control (P≤0.05)

Figure 2 shows the typical effects of 0.1 to 1.0 mM L-DOPA on soybean roots. There are noticeable differences in the roots: in particular, the elongation of the primary root of the treated seedlings was inhibited by the allelochemical. Furthermore, the roots became black, thicker, and less flexible.

Effects of L-DOPA on Antioxidant Enzymes The antioxidant enzymes activities in the roots of seedlings treated with L-DOPA were significantly different from those of controls (Fig. 3). The data revealed that 0.1 to 1.0 mM L-DOPA increased the SOD activities from 35% to 190%, in comparison with the control (Fig. 3a). POD activities also were significantly increased by approximately 43%, regardless of the L-DOPA concentration, when compared to the control (Fig. 3b). In contrast, CAT activities were reduced by approximately 40% after the exposure of 0.1 to 1.0 mM L-DOPA, when compared to the control (Fig. 3c).

Effects of L-DOPA on ROS and Lipid Peroxidation The exposure of soybean seedlings from 0.1 to 1.0 mM L-DOPA significantly reduced the $O_2^{\bullet-}$ contents from 63% to 37% (Fig. 4a) and the H_2O_2 contents from 66% to 80% (Fig. 4b), respectively, in comparison with the control. Lastly, the data revealed that the levels of lipid peroxidation decreased from 46% (at 0.1 mM L-DOPA) to 66% (at 1.0 mM L-DOPA), with respect to the control (Fig. 4c).

Discussion

Overall, our results reveal that L-DOPA increased PPO activity and melanin production at all of the concentrations

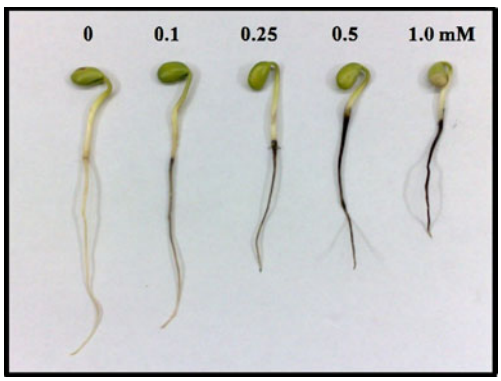


Fig. 2 A comparison of root coloration in non-treated (0 mM) and 0.1 to 1.0 mM L-DOPA-treated soybean seedlings

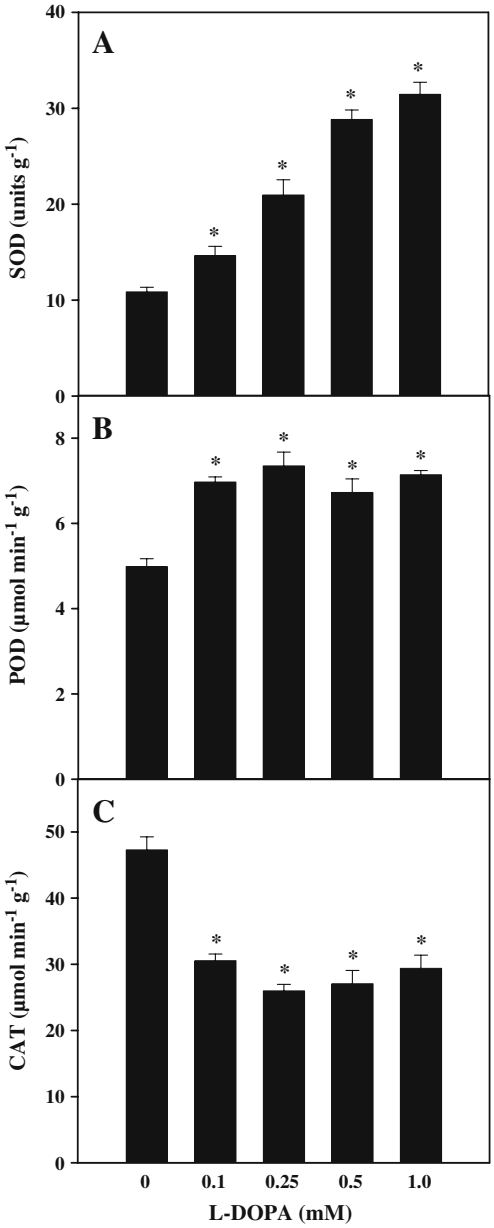


Fig. 3 The effects of different concentrations of L-DOPA on the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in soybean roots. *Values (N=3±SE) differ statistically (Dunnett's multiple comparison test) from the control ($P \leq 0.05$)

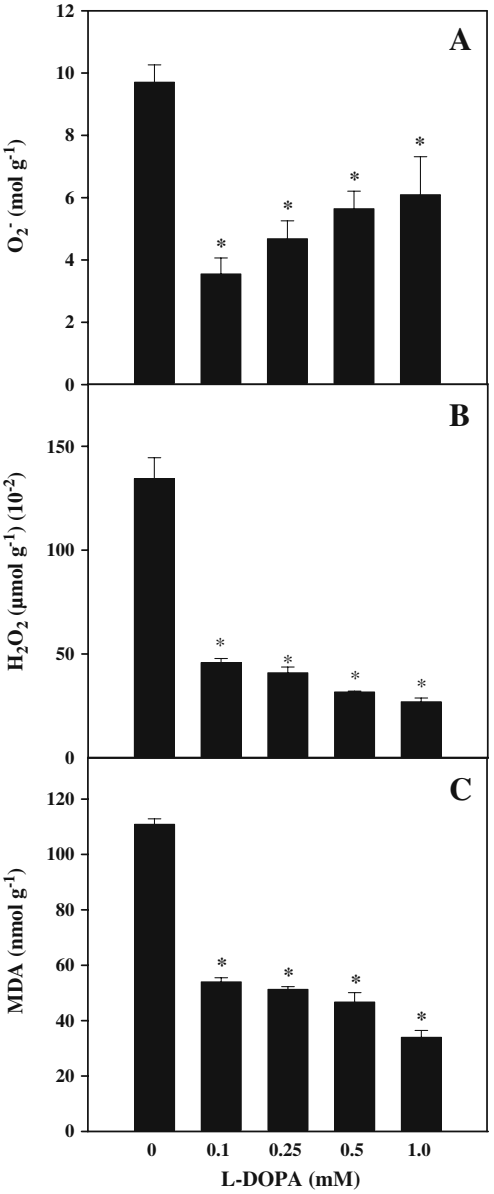


Fig. 4 The effects of different concentrations of L-DOPA on the contents of superoxide anion $\left(O_2^{\bullet -}\right)$ and hydrogen peroxide (H_2O_2) and the levels of lipid peroxidation in soybean roots. *Values (N=3± SE) differ statistically (Dunnett's multiple comparison test) from the control ($P \leq 0.05$)

tested (Fig. 1). The activities of both SOD and POD increased and were accompanied by a decrease in the $O_2^{\bullet -}$ and H_2O_2 contents; these results also were associated with a significant reduction in the levels of lipid peroxidation (Figs. 3 and 4). The only exception, with regards to the tested oxidative factors in the roots, was CAT activity, which was substantially lower after L-DOPA treatments (Fig. 3c).

Reduction of root growth by L-DOPA has been reported in several plant species and it has been considered as an allelopathic effect of this phenolic non-protein amino acid (Hachinohe et al., 2004; Nishihara et al., 2004; Topal and Kocaçalişkan, 2006; Hachinohe and Matsumoto, 2007b; Soares et al., 2007). After absorption, L-DOPA can be metabolized to melanin by enzymatic reactions (Pattison et al., 2002). Both PPO and POD are active enzymes in the production of pigments in damaged plant tissues (Albrecht and Kohlenbach, 1990). PPO is a copper protein that uses molecular oxygen to catalyze the oxidation of monophenols to *o*-diphenols and further to *o*-quinones (Muñoz-Muñoz et al., 2010); POD mediates the oxidation of L-DOPA to form dopaquinone and melanin (Nishihara et al., 2004). By

evaluating the response of seed germination of different plant species to the allelochemical, L-DOPA, Nishihara et al. (2004) confirmed a relationship between the changes of color on filter paper and the inhibition of radical growth. They verified that the radicle became shorter and turned black or light brown in L-DOPA-treated seedlings, when compared to controls. Using carrot (*Daucus carota* L.), Hachinohe and Matsumoto (2007a) have shown that the reduction of cell growth and melanin accumulation is dependent on the L-DOPA concentration. Our results showed that the significant increase in the PPO (Fig. 1a) and POD (Fig. 3b) activities was associated with an expressive browning of the roots (Fig. 2), thus, providing evidence for the active synthesis of melanin from the oxidation of L-DOPA (Fig. 1b).

Hachinohe and Matsumoto (2007b) reported that the amount of melanin accumulated in lettuce roots correlated with the growth inhibitory activity of L-DOPA. We have not found, however, a good correlation between the increased melanin concentration in soybean roots and the degree of root growth inhibition (r^2=0.64, P=0.104, regression analysis; data not shown). This finding indicates that melanin is not the only cytotoxic substance responsible to inhibitory effects on soybean roots.

It has been suggested that L-DOPA generates ROS ($O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$) or semiquinone radicals and quinones during the production of melanin (Pattison et al., 2002). In general, ROS and quinones can interact with cellular components, triggering stresses in plants, damaging membranes, blocking nutrient uptake, and hindering the root growth (Gill and Tuteja, 2010). In this context, Hachinohe and Matsumoto (2007b) evaluated whether the phytotoxicity of L-DOPA was due to the ROS formed during its oxidation to melanin. Those authors reported that ascorbic acid and α-tocopherol reduced the observed oxidative damage, alleviating the suppression of lettuce root growth that was caused by L-DOPA and preventing the accumulation of melanin. However, they did not quantify the ROS species that were formed during the oxidation of L-DOPA *in vivo*, even though ROS generation has been verified *in vitro* by electron spin resonance (Kruk et al., 1999). Our results contradict those described by Hachinohe and Matsumoto (2007b) since the levels of ROS species ($O_2^{\bullet-}$, H_2O_2) decreased in soybean roots treated with L-DOPA, when compared to controls (Fig. 4). A possible explanation for this might be that different plant species operate different mechanisms to protect cells from L-DOPA effects, as suggested by Nishihara et al. (2004). In agreement, Hachinohe et al. (2004) demonstrated significant differences in sensitivity to L-DOPA among many plant species, including barnyardgrass (*Echinochloa crus-galli*) and lettuce, tolerant and susceptible species, respectively. In this regard, the levels of melanin and thiobarbituric acid reactive substances (TBARS) increased remarkably in L-DOPA-treated lettuce roots, but not in barnyardgrass (Hachinohe and Matsumoto, 2005). Thus, we presume that the phytotoxicity of L-DOPA in soybean roots is not due to oxidative stress caused by ROS generated from the melanin synthesis pathway.

Several studies have reported an antioxidant activity of L-DOPA. For example, a direct role as a scavenger of free radicals and as a modulator of cellular antioxidant enzymes has been postulated (Randhir et al., 2006). Gülçin (2007) investigated the antioxidant properties of L-DOPA by using butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol, and Trolox as references. In that study, it was reported that L-DOPA inhibited 68% of the lipid peroxidation, whereas BHA, BHT, α-tocopherol, and Trolox inhibited 74, 71, 55, and 20% of the linoleic acid peroxidation, respectively. Interestingly, some studies have also suggested an antioxidant role for melanin in animals (Wang et al., 2006; Tu et al., 2009). Using *in vitro* bioassays, Tada et al. (2010) verified that melanin potentially interacted with $O_2^{\bullet-}$ and $^1O_2$, thus, scavenging these free radicals. However, this possible antioxidant role of melanin in plants deserves more studies.

The SOD, POD, and CAT enzymes are well-known scavengers of ROS. SOD acts as the first line of defense against ROS, dismutating $O_2^{\bullet-}$ to H_2O_2. POD and CAT also are indispensable for this defense mechanism because, subsequent to the action of SOD, both enzymes detoxify H_2O_2 to H_2O (Apel and Hirt, 2004). In the current study, SOD and POD activities were significantly increased following L-DOPA exposures (Fig. 3a and b), and these increases were associated with decreases in the levels of $O_2^{\bullet-}$ and H_2O_2 (Fig. 4a and b). The observation that these ROS species were reduced was corroborated by the decrease in the levels of lipid peroxidation (Fig. 4c).

Another important fact is that the content of H_2O_2 is reduced during the synthesis of lignin at the cell wall. Soares et al. (2007) have observed a significant increase in the cell wall-bound POD activity involved in the lignification of soybean roots treated with the phenolic amino acid L-DOPA. In fact, lignification requires oxidative coupling and is dependent on H_2O_2. In the present study, because the POD activities increased (Fig. 3b), the decline in H_2O_2 that was observed after the L-DOPA treatments (Fig. 4b) is also in agreement with the reduced activities of CAT (Fig. 3c). This may be explained by the fact that H_2O_2 is decomposed by POD during the lignin synthesis, as suggested by Soares et al. (2007). Furthermore, these results are also consistent with previous research, because other phenolic allelochemicals, such as *p*-coumaric and caffeic acids have been shown to increase POD activity associated to the decrease in H_2O_2 levels (Zanardo et al., 2009; Bubna et al., 2011). It is possible, therefore, that the reduction in CAT activity

(Fig. 3c) is due to low levels of H_2O_2 in soybean roots treated with L-DOPA.

This combination of findings provides some support for the premise that there is a coordinated and precise balance between SOD, POD, and CAT activities that suppress the ROS levels. In general, when one of these enzymes is reduced, others are upregulated (Apel and Hirt, 2004). From all these observations, we suggest that the antioxidant enzymatic system is directly involved in the reduction of ROS levels. In addition, the possible antioxidant properties of L-DOPA or melanin cannot be ruled out, since the production of ROS did not increase, but rather decreased below the control in the presence of L-DOPA (Fig. 4a and b).

The striking fact revealed in the current study is that, at least for soybean, the phytotoxicity of L-DOPA seems not due simply to ROS species generated during its oxidation to melanin, as hypothesized by Hachinohe and Matsumoto (2007b). Moreover, we found additional experimental support for our previous hypothesis that an excessive production of lignin and premature lignification of the cell walls is an important mode of action of L-DOPA in soybean (Soares et al., 2007).

More recently, Golisz et al. (2011) used DNA microarrays to analyze the gene expression profiles of *Arabidopsis thaliana* exposed to L-DOPA. The authors verified the expression of 179 genes and suggested that the response of plants to L-DOPA can affect genes involved in amino acid metabolism, oxidative stress, melanin synthesis, lipoperoxides, phenolic compounds, and lignification. Regarding the latter, a future study investigating the gene expression profiles of soybean roots exposed to L-DOPA would be interesting. This approach may lead to a better understanding of the mode of action of L-DOPA in plants, and an allelopathic role in an agroecological system, especially with regard to reducing the use of synthetic herbicides.

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(Cardé and Willis, 2008), odors from different plumes are not homogeneously mixed when plumes come into contact. This may facilitate discrimination of relevant filaments from background semiochemicals. The size and shape of the odor source, as well as air turbulence, are major factors that determine the structure of the plume (Murlis et al., 1992, 2000; Riffell et al., 2008). In addition, the odor concentration in the plume decreases with downwind distance (Baker and Haynes, 1989; Murlis et al., 2000) but, due to the filamentous structure, some odor packages may remain relatively undiluted far away from the source (Murlis and Jones, 1981; Cardé and Willis, 2008).

In this study, we conducted a series of field trapping experiments in which the responses of the spruce bark beetle, *Ips typographus* (Coleoptera, Curculionidae, Scolytinae), and the Egyptian cotton leaf worm, *Spodoptera littoralis* (Lepidoptera, Noctuidae), to separation of different odor sources were compared. Concurrently, plume structure and overlap were measured. The structure of an odor plume may be measured in detail using equipment that is highly sensitive, with a high sampling rate (Murlis and Jones, 1981; Riffell et al., 2008), or by using inexpensive smoke or soap-bubble generators, which more easily visualize plumes (David et al., 1983). Several studies have investigated odor plume structure, and the mechanisms by which insects navigate in plumes (reviewed in e.g., Cardé and Willis, 2008). However, the manner in which plumes from different sources interact (overlap) physically, and how different insects discriminate between them, are less known. Plume discrimination studies have focused mainly on the responses of male moths to female sex pheromone and pheromone antagonists. These studies show great plume discrimination ability by moths. Spacing between components, by as little as a few millimeters, can influence pheromone attraction, and simultaneous perception of an antagonist is required to suppress pheromone source location (Witzgall and Priesner, 1991; Rumbo et al., 1993; Valeur and Löfstedt, 1996; Fadamiro et al., 1999). Less is known about the responses of non-lepidopteran insects to overlapping odor plumes, although two studies on *Dendroctonus* bark beetles demonstrated synergistic pheromone component interactions over source spacing distances of several meters (Byers, 1987; Sullivan and Mori, 2009).

Male and female *I. typographus* are attracted to the male-produced aggregation pheromone, a mixture of (4*S*)-*cis*-verbenol (cV) and 2-methyl-3-buten-2-ol (MB), which is released when a suitable host tree is located. Both components are necessary for attraction (Schlyter et al., 1987a). Pheromone attraction is modulated by anti-attractive, non-host volatiles (NHV) from angiosperm plants (Zhang and Schlyter, 2004). A strong, synergistic effect of inhibition is obtained by a combination of green leaf volatile (GLV) alcohols, C_8-alcohols, *trans*-conophthorin, and verbenone (Zhang and Schlyter, 2003). 1,8-Cineole, a monoterpene abundant in attacked spruce, also suppresses pheromone attraction (Andersson et al., 2010).

The female sex pheromone of *S. littoralis* consists of (*Z,E*)-9,11-tetradecadienyl acetate (Z9E11-14Ac; major component) and (*Z,E*)-9,12-tetradecadienyl acetate (Z9E12-14Ac; minor component), (Kehat et al., 1976). The monoene, (*Z*)-9-tetradecenyl acetate (Z9-14:Ac), is found in low amounts in the pheromone gland (Nesbitt et al., 1973), but inhibits attraction to pheromone at high doses (Campion et al., 1980).

In this paper, we report how trap catches of *I. typographus* and *S. littoralis* to their respective pheromones are affected by spacing between pheromone components, as well as by spacing between the pheromone and behavioral inhibitors. As the beetle colonizes both standing and fallen trees, spatial separation was performed both vertically and horizontally. Apart from using point sources, we also studied the effect of an artificially created non-host odor background, corresponding to a larger scale "habitat odor" concept (Zhang and Schlyter, 2003; Jactel et al., 2011), on bark beetle trap catches. Our hypotheses were: (*a*) At the scale of the trapping devices used in our system, overlap of two plumes will depend on the distance between the sources; (*b*) Spacing of odor sources will modulate the behavioral responses of the two species over similar distances; (*c*) The scale of responses to spaced attractant sources and spaced attractant and anti-attractant sources is the same; (*d*) A more homogeneous background of anti-attractant will give a stronger reduction of attraction than will discrete sources. Our experiments were designed to mimic natural or semi-natural situations, and revealed not only differences between species, but also provided information that may be used to develop efficient ways to use semiochemicals for disruption of host and/or mate finding in the two pest insects.

Methods and Materials

Odor Plume Measurements and Visualizations Odor plume concentrations were measured under semi-field conditions (at SLU campus, Alnarp, Sweden) with a photo ionization detector (PID; ppbRAE + model PGM7240, equipped with a 10.6 eV lamp, RAE systems Inc. San Jose, CA, USA). The detector measures concentrations to the low ppb range. Three open PE #733 vials (20 mm diam. opening, Kartell, Italy), filled with 5 ml hexane, were used as odor sources, as the dispensers in the trapping experiments released too low concentrations of volatiles to be detected. Dispensers were placed under an inverted 250 ml grey plastic cup (as in the *Ips* experiments, described below), positioned 90 cm above ground, a similar height as in our *Ips* experiments.

The PID inlet was 90 cm above ground, and plume concentration data were collected at 1 Hz, over 2 min, at various distances downwind. The detector was kept stationary during a replicate, irrespective of changes in wind direction. The wind speed was <0.5 m/s.

For longer distances, plume width, as well as vertical and horizontal overlap of plumes, were visualized and estimated using soap bubble generators (Gazillion bubble machine, item # 32417, Funrise Inc., China) hung on Lindgren traps, positioned like the dispensers in the vertical and horizontal trapping experiments with *Ips* (see below). The experiment was performed on a lawn, with ca 50 m to the nearest tree, at SLU, Alnarp. Measurements were taken on 3 d (i.e., replicates), and average wind speeds recorded for 30 sec intervals. Because of variable wind speed and wind direction, two or three estimates of plume width and overlap were recorded: typical, maximum, and/or minimum. Accuracy of plume estimates was aided by equally spaced marked poles in the ground at various distances downwind from the trap. Estimates were taken when bubbles exited the generators in the same direction as the wind flow. Video recordings confirmed visual estimates.

Spacing Experiments with Ips, General Design Field trapping experiments with *I. typographus* were conducted 3 May–3 June 2008 at Sporrakulla (56° 16′34″ N, 14° 15′20″ E) and Parismåla (56° 34′50″ N, 15° 28′56″ E), both in south Sweden. Treatment positions were initially randomized, and changed after each replicate, so that each treatment appeared in each location at least once (i.e., temporal Latin square design). Beetle collection and dispenser rotation were carried out when ≥100 beetles were caught by all traps collectively. A replicate lasted from one to a few hours, depending on flight activity. Dispensers were put under an inverted 250 ml grey plastic cup. Traps were separated by at least 15 m. Table 1 lists chemicals, dispensers, and release rates used in all *I. typographus* and *S. littoralis* experiments.

Spacing Between Pheromone Components of Ips MB and cV were added to separate dispensers and used in the vertical and horizontal pheromone source spacing experiments. Lindgren multiple-funnel traps, extended to 19-funnel size (height ca 160 cm, diam. 19 cm), were used to study the effect of vertical separation of the two components at 16, 32, 48, 80, and 112 cm (Supplementary Material, Fig. S1-A). The cV and MB dispensers were spaced at equal distances in opposite directions from the central funnel. For each spacing distance, two traps were used: one with cV moved upwards and MB downwards, and another with the opposite pattern. Controls were, blank, only MB, only cV, and both components at 0 cm spacing.

In the horizontal experiment, Lindgren traps (5-funnel size) were connected to lightweight wind vanes (designed and manufactured by company Fa: J. Lantz, Smedstorp, Sweden) placed on tripods. Wind vanes were used to minimize variation in trap catch and to ensure a constant distance between plumes, despite changes in wind direction (Supplementary Material, Video 1). An aluminum rod, on which dispensers were hung, was fixed horizontally at the central funnel, at a right angle compared to the wind vane (and thus to wind direction). In treatments with the components separated by 16, 32, 48, and 80 cm, dispensers for cVand MB were positioned at opposite directions, away from the center of the trap. The positions (i.e., right or left arm) were switched between replicates to control for directional effects. Controls were, MB, cV, and both components at 0 cm separation.

Spacing Between Pheromone Components of Spodoptera The effect of horizontal pheromone component spacing on *S. littoralis* males was studied in a cotton field, 25 km north of the city of Assiut, middle Egypt in 2009 (27° 10′58″N, 31° 10′58″ E). Z9E11-14Ac and Z9E12-14Ac were tested at three doses (5:0.05, 50:0.5, and 500:5 μg, respectively; Table 1), and spaced 2, 3, 4, 8, and 16 cm apart. Half of the traps (Economic green funnel trap, Oecos, Hertfordshire, UK; height 22 cm, diam. 13 cm) that contained both components had the major component in the center and the minor moved outwards, and the other half of the traps *vice versa*. Dispensers outside the trap were shaded from sunlight (Supplementary Material, Fig. S1-B). Z9E11-14Ac or Z9E12-14Ac alone, 0 cm spacing of both components, and a blank trap were controls. Traps were placed in the cotton canopy with an inter-trap distance of 10 m. Two separate experiments were run. Experiment 1 was performed from July 25 to August 12, and Experiment 2, in which the natural population was boosted with 450 laboratory-reared pupae, was run 6–24 September. Catches were checked, and treatments rotated (= one replicate) two trap positions every second day. For practical reasons, wind-oriented traps could not be used for *Spodoptera*.

Spacing Between Pheromone and Anti-attractant of Ips In two (vertical and horizontal source spacing) experiments, the commercial IT ECOLURE Tubus aggregation pheromone dispenser (Fytofarm Ltd., Bratislava, Slovakia) was used in combination with an anti-attractive NHV cocktail (*trans*-conophthorin, 3-octanol, 1-octen-3-ol, 1-hexanol, 1,8-cineole, and verbenone Zhang and Schlyter 2004; Andersson et al. 2010; individual compounds in separate dispensers; Table 1). In the vertical experiment, the same experimental design, traps, and spacing distances as in the analogous pheromone component spacing experiment were used. A blank trap, a trap with 0 cm between pheromone and NHV sources, and a pheromone-only trap were controls. The design of the horizontal experiment was

Table 1 Chemicals, release rates, and dispensers

Compound	Source	Purity (%)	Release rate (mg/day ± 95% CI)[a]	Amount	Dispenser
(*Z,E*)-9,11-tetradecadienyl acetate	Pherobank, The Netherlands	98		5–500 μg	Red rubber septa[b]
(*Z,E*)-9,12-tetradecadienyl acetate	Pherobank, The Netherlands	98		0.05–5 μg	Red rubber septa
(*Z*)-9-tetradecenyl acetate	Pherobank, The Netherlands	96		2.5–250 μg	Red rubber septa
2-methyl-3-buten-2-ol	Aldrich, USA	97	57±0.8[d]	2 ml	#733 PE[c] vial, 2 mm diam. hole in lid
(−)-cis-verbenol	Borregaard, Norway	95	1±0.05[d]	40 mg	2 ml hard PE vial, 9 mm diam. hole in lid
IT ECOLURE Tubus	Fytofarm Ltd, Slovakia		95±0.3		Closed PE tube
IT-REP	Fytofarm Ltd, Slovakia		40±6.2		Wick-aluminum protected dispenser
1-hexanol	Aldrich, USA	98	4.1±0.13[e]	200 μl	#730 PE vial, open 6 mm diam.
(±)-3-octanol	Acros, USA	99	2–3[f]	150 μl	#731 PE vial, 8 mm diam. hole in lid
(±)-1-octen-3-ol	Acros, USA	98	2–3[f]	150 μl	#731 PE vial, 8 mm diam. hole in lid
(−)-verbenone	Aldrich, USA	99	1.1±0.03[d]	2×150 μl	Two #730 PE vials, open 6 mm diam.
(±)-*trans*-conophthorin	Pherotech, Canada	98	0.5±0.04	50 μl	2 capillaries, 1 mm diameter
(±)-1,8-cineole	Aldrich, USA	99	18±0.76	300 μl	#730 PE vial, closed
Flakes, verbenone	Hercon Environmental USA		4.2±0.7[g]	20 g flakes	
Flakes, hexanol + *trans*-conophthorin	Hercon Environmental USA		7.9±3.2[g]	20 g flakes	

[a] Release rates measured by gravimetric methods in the laboratory in this study at 22±1°C and 0.5 m/s wind speed, unless otherwise indicated

[b] PheroNet AB, Lund, Sweden

[c] Polyethylene vial (Kartell, Italy)

[d] From Schlyter et al. (1989)

[e] From Zhang et al. (1999)

[f] From Zhang and Schlyter (2003), mixed in the same vial

[g] Release rate for 1 g flakes/day

similar to the horizontal pheromone component experiment, and the same traps were used. The difference was that the attractant was always placed in the center of the trap to ensure that attracted beetles were caught. Control traps were as in the vertical pheromone/NHV test.

Spacing Between Pheromone and Anti-attractant of Spodoptera The effect of separating the reported (Campion et al., 1980) pheromone antagonist, Z9-14Ac, from the pheromone (Z9E11-14Ac: Z9E12-14Ac; 500:5 μg), was studied in an experimental field, ca 4.5 km north-east of Assiut, Egypt, 11–29 September 2009, using a similar experimental design as in the pheromone component separation experiment. The pheromone source was placed in the center of the trap and the antagonist placed outwards, 2, 3, 4, 8, and 16 cm away from it. Three doses of Z9-14Ac were tested (2.5, 25, and 250 μg). A blank trap, a pheromone-only trap, and 0 cm distance between pheromone and antagonist sources were controls.

Pheromone Attraction of Ips in a Background of Anti-attractant The effect of an artificial NHV "background" on catches of beetles in pipe traps (Zhang and Schlyter, 2003) baited with aggregation pheromone (IT-ECOLURE) was tested during 27–30 April and 30–31 May 2009, in Skillingaryd (57° 28′39″ N, 14° 7′58″ E), south Sweden. We used a randomized block design with 8–10 blocks. Treatment positions were maintained throughout the experiment. NHV background was created by placing semi-commercial *I. typographus* repellent IT-REP (Fytofarm Ltd; 1-hexanol, *trans*-conophthorin, 3-octanol, 1-octen-3-ol, and verbenone as active chemicals) dispensers at eight positions (at ca. 1.2 m height) in a ring around a central pheromone trap (Supplementary Material, Fig. S1-C). Three separate experiments were performed, using a ring of NHV dispensers at radii of 1, 2, and 3 m, respectively, around the pheromone trap. Two NHV doses were tested: 1 (low) or 3 (high) dispensers per position.

In an additional experiment, a more uniform NHV background was created by using NHV-containing flakes (Hercon Environmental, Emigsville, PA, USA). Flakes baited with verbenone (Gillette et al., 2009) or 1-hexanol plus *trans*-conophthorin, were used in combination (50:50 weight: weight ratio). A total of 40 g (ca 6000 flakes) were distributed on the ground around the pheromone trap, covering an area with a 2 m radius. The release of active

compounds from this amount corresponded to the release of eight IT-REP dispensers, as calculated from weight-loss experiments and odor collections in the laboratory. Hence, 8 IT-REP dispensers at 1 m distance were a positive control. A control test in which the IT-REP dispenser was placed in the trap with the pheromone confirmed the activity of the semi-commercial repellent.

Data Analysis Regressions were used to analyze how plume width and overlap changed with downwind distance.

In the vertical spacing experiments with *Ips*, we found no difference between catches regardless of which dispenser was positioned above or below the other. Therefore, we pooled catches from these treatments by calculating the average catch per replicate in each trap pair. Similarly, there was no difference between the two horizontal pheromone component spacing experiments (i.e., 25 July–12 August, and 6–24 September, respectively) with *Spodoptera,* and these data were also pooled, doubling the number of replicates.

As trap catches consist of count data that usually do not fulfill all assumptions for testing by General Linear Models, absolute catches were analyzed by Generalized Linear Models (*GzLM*), using the Poisson distribution as model (Hoffmann, 2004). However, in the bark beetle spacing experiments, goodness of fit analyses and likelihood ratio tests demonstrated over-dispersion (i.e., variances greater than the means), and that the negative binomial distribution with log link (SPSS 16.0) was better suited as model. In all tests, Pearson χ^2 was used to obtain variance estimates and significance levels. The *Sidak* test was used in pair-wise comparisons.

Traditionally, data from field tests similar to ours have been analyzed by *ANOVA*, typically after some transformation to achieve normality and homoscedasticity (e.g., Zhang and Schlyter, 2003; Reeve and Strom, 2004). For comparison with our *GzLMs*, we also analyzed our data with factorial *ANOVA* on, untransformed catches in the *Spodoptera* tests, catch proportions (p) within replicates for the NHV background experiments with *Ips*, and arcsin $p^{0.5}$ transformed catches for the *Ips* odor spacing experiments. *GzLMs* and *ANOVAs* always gave numerically similar *P*-values and did not differ in terms of acceptance or rejection of tested null hypotheses. Thus, for data sets in which we tested both, we only report the *GzLM* statistics. Pair-wise comparisons were done with *t-tests* or, alternatively, *Mann–Whitney U tests* in case of unequal variances. *Ips* sex ratios were analyzed with 95% binomial confidence intervals (CI) (Newcombe, 1998). All tests were performed using SPSS 16.0 at α=0.05.

Hedges' g standardized unbiased effect sizes (Hedges and Olkin, 1985) were calculated for data from all field-trapping experiments. The effect size is a unit-less measure of a treatment effect, obtained by dividing the difference between the means of the control and treatment with the pooled standard deviation for those means (Nakagawa and Cuthill, 2007). The effect size measure facilitates comparisons between different studies or insect systems. For *I. typographus*, effect sizes were calculated from catch proportions, rather than raw numbers caught, as replicates differed widely in absolute catch. An effect size above 0.8 is often regarded as a large effect (Nakagawa and Cuthill, 2007).

Results

Odor Plume Measurements and Visualizations Photo ionization detector (PID) measurements taken downwind of hexane dispensers (similar to those in the *Ips* experiments) showed responses up to a distance of 2 m (Fig. 1a). Maximum concentration per odor burst (Fig. 1b) and the frequency of detected bursts (Fig. 1a) decreased with downwind distance from source. The response of the PID to background odors was zero ppb.

The pattern of bubbles produced by the soap bubble generator attached to a Lindgren trap showed that odor plume width increased linearly during downwind displacement (Fig. 2a). In general, wind speed and direction were highly variable, which sometimes made it difficult to obtain accurate estimates. The 'minimum' estimates were observed typically during gusts of higher than average wind speed. Average wind speed (during 30 sec) ranged from 0.3 to 7.3 m.sec^{-1}, with an overall mean of 3.1 m.sec^{-1}. Occasionally, at low wind speed, soap bubbles went straight up, and on other occasions reached ground a few meters downwind, indicating variation in vertical air movement, although gravity may have been responsible for the soap bubble descent.

Attaching a second bubble generator to the trap showed that the downwind distance at which the plumes started to overlap increased linearly with vertical or horizontal distance between generators (Fig. 2b, c). In general, plume overlap started farther downwind with increasing wind speed, but we also observed large variation during periods of relatively unchanged wind speed, especially with larger source spacing (Supplementary Material, Video 2 and 3). For the 'typical' measurement, the horizontal and vertical spacing showed similar patterns of plume overlap.

Spacing Between Pheromone Components of Ips Separating the cV and MB reduced trap catch, with the effect being distance dependent (Fig. 3a, b). The response to spacing was similar in the vertical and horizontal tests (i.e., no interaction between spacing distance and direction; $\chi^2_4 = 1.8$, $P>0.05$). Having the pheromone components spaced 16 cm apart resulted in as many beetles caught as

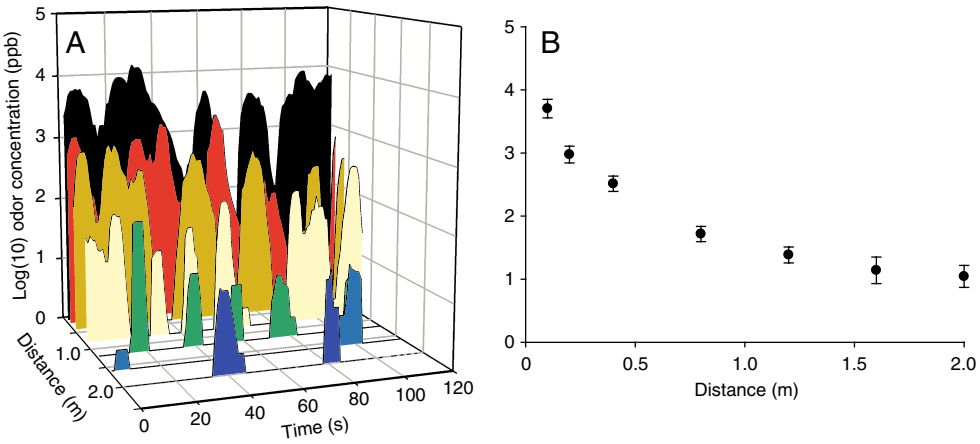


Fig. 1 Plume characteristics with respect to downwind distance from an odor source, as measured by a photo ionization detector (*PID*). **a** Raw PID data over a 2 min replicate, illustrating spatial and temporal changes in odor concentration. Fewer bursts and lower concentrations were detected further downwind. The position of the detector during a replicate was kept constant. **b** Mean maximum concentration per odor burst (N=3). *Error bars* show SEM. Note: the drop in concentration with downwind distance may be amplified by the low sampling rate (1 Hz) of the detector, as compared to previous studies (Murlis and Jones, 1981; Murlis et al., 2000; Justus et al., 2002)

when the components were together (*Hedges' g* effect size close to zero). In the vertical test, 112 cm spacing caught more beetles (*GzLM*: $\chi^2_3 = 52.6$, $P<0.001$, followed by the *Sidak post-hoc* test) than the negative controls (blank, cV, or MB alone). None of the pheromone components alone caught more beetles than the blank. In the horizontal test, 80 cm between pheromone components caught more beetles ($P<0.05$) than cV alone, and almost more than MB alone ($P=0.074$) (*GzLM*: $\chi^2_2 = 16.9$, $P<0.001$, followed by the *Sidak* test). Thus, in both experiments, a large spacing of pheromone components did not fully shut down bark beetle trap catch.

In the horizontal test, the percentage of males (27–43%) caught increased with spacing distance (0–48 cm), as analyzed by 95% binomial CI. However, at 80 cm spacing, the percentage of males caught was only 35%. The percentage of males caught did not differ between treatments in the vertical test.

Spacing Between Pheromone Components of Spodoptera We found a general pattern of increased spacing between components resulting in reduced male *S. littoralis* trap capture. However, whether the major compound was positioned in the center of the trap (Fig. 3c) or moved outwards (Fig. 3d) had an effect on trap capture ($\chi^2_1 = 41.8$, $P<0.001$, $g=0.42$). This effect was dependent on spacing distance (interaction between position of major compound and spacing distance, *GzLM*: $\chi^2_4 = 34.2$, $P<0.001$). More males were caught when the major compound was positioned in the center (mean catch ± SD: 1.4±1.3) as compared to when the minor compound was positioned there (0.9±1.0). At the 16 cm-spacing distance (Fig. 3c–d), the effect of position was especially strong, as shown by a g-value of 1.69. Data were subsequently analyzed separately, with respect to the position of the major compound, to test for the effect of spacing. *GzLM* demonstrated effects of both spacing distance and pheromone dose on male trap catch, irrespective of the position of the major compound (Fig. 3c–d). A strong effect ($g<-1.0$) of spacing was observed already at 2 cm between compounds. Higher doses caught more moths, especially when the compounds were positioned close together. The 0 cm spacing attracted more males than all other treatments ($P<0.001$).

Compared to treatments with the major component in the center, traps with the major component alone caught more moths than the two components spaced 4–8 cm (*GzLM* followed by the *Sidak* test: $P<0.001$). Catch to the major component alone was not different from that to the 2 cm and 16 cm spacing distances (Fig. 3c). Traps with the minor component alone caught more males than those with both components spaced 8 cm ($P<0.05$). Compared to the treatments with the minor component in the center, traps with the major component alone caught more males than those with both components spaced 3–16 cm ($P<0.01$–$P<0.001$; Fig. 3d). Catches to the 16 cm spacing were, in this case, not different from the minor component alone.

Spacing Between Pheromone and Anti-attractant of Ips Spacing distance between the pheromone and the NHV blend had a strong effect on beetle trap catch (Fig. 4a, b). In the vertical experiment, the catch in the 112 cm spacing treatment was still lower ($t_{30}=3.46$, $P<0.01$) than that in the pheromone control and, along with the large effect size ($g=-1.2$), this indicated that the inhibitory effect of NHV reached beyond a separation of 112 cm. In the horizontal test, however, the 80 cm spacing caught similar numbers of

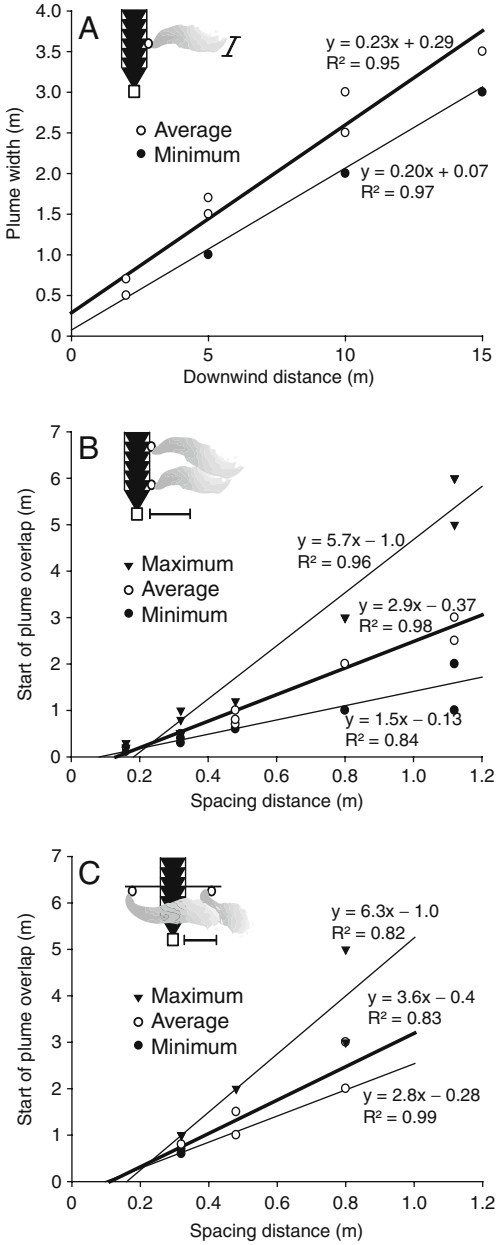


Fig. 2 **a** Relationship between plume width and downwind distance from a single bubble generator. Effect of, **b** vertical, or **c** horizontal spacing between two bubble generators on the downwind distance at which plume overlap started. N=3

beetles as the pheromone control (t_{26}=0.38, P>0.05). Otherwise, the response to increased spacing was similar between the vertical and horizontal tests, as shown by the absence of an interaction between spacing distance and direction ($\chi^2_4 = 1.6$, P>0.05). In both experiments, NHV at 0 cm spacing from the pheromone resulted in greatly reduced trap catch (g=−2.6 and g=−2.1 for vertical and horizontal test, respectively), but the catch was larger than that in blank traps (vertical: z=−4.30, P<0.001; horizontal: z=−4.81, P<0.001).

In the vertical test, the percentage of captured males was reduced by the presence of NHV, compared to pheromone alone (27–32% males in NHV treated traps, 35% in the pheromone alone trap). A similar, but non-significant, tendency was found in the horizontal test (24–31% in NHV traps, 34% in the pheromone alone trap). Spacing distance, as such, had no effect on the percentage of males captured.

Spacing Between Pheromone and Anti-attractant of Spodoptera The anti-attractant, Z9-14Ac, modulated trap catch differently, depending on dose (Fig. 4c). Only at the high dose (250 μg) was it antagonistic when positioned at the same point (g=−1.7) as the pheromone (*GzLM* followed by the *Sidak* post hoc test: P<0.05). In fact, the lowest dose (2.5 μg) elicited increased male catch (P<0.05, g=1.6), whereas the medium dose (25 μg) had no influence. Furthermore, *GzLM* demonstrated that the effect of spacing distance between pheromone and antagonist was dose-dependent, as shown by the interaction between dose and distance (Fig. 4c). Analyzing the doses separately, showed an effect of anti-attractant spacing at the low ($\chi^2_5 = 12.0$, P<0.05) and high ($\chi^2_5 = 23.4$, P<0.001) doses, with increased spacing reducing and increasing trap catch, respectively. Spacing distance at the intermediate dose did not affect trap catch ($\chi^2_5 = 3.2$, P>0.05).

Pheromone Attraction of Ips in a Background of Anti-attractant A control experiment verified the strong effect of the IT-REP dispensers, as one dispenser positioned inside the pheromone trap caught, on average, 6.5% of the beetles within a replicate, compared to the pheromone alone control, which caught 93.5% (g=−14.8 based on catch proportions within replicates; N=6). Pheromone trap catch was reduced by a background of NHV, released from IT-REP dispensers at eight positions around the trap. Factorial *ANOVA* showed an interaction between treatment and distance between NHV and pheromone (Fig. 5a). Thus, separation distances were further analyzed, separately, using one-way *ANOVAs*. Dispensers, both at 1 m and 2 m from the trap, reduced pheromone attraction (1 m distance: $F_{2,\ 21}$=36.8, P<0.001, g<−3.0; 2 m distance: $F_{2,\ 27}$=16.0, P<0.001, g<−1.7; Fig. 5a); there was no difference between the low and high NHV dose. At the 3 m distance, we found no difference between treatments ($F_{2,\ 21}$=2.2, P> 0.05), although a tendency for reduced-catch effects of NHV was apparent, as shown by the relatively large effect size (g≈−0.8).

The more uniform NHV background, created by ca 6000 flakes, also caused reduced pheromone trap catch (Fig. 5b). The number of beetles caught by traps surrounded by flakes was lower than that caught by the pheromone control, but not different from that caught by the negative

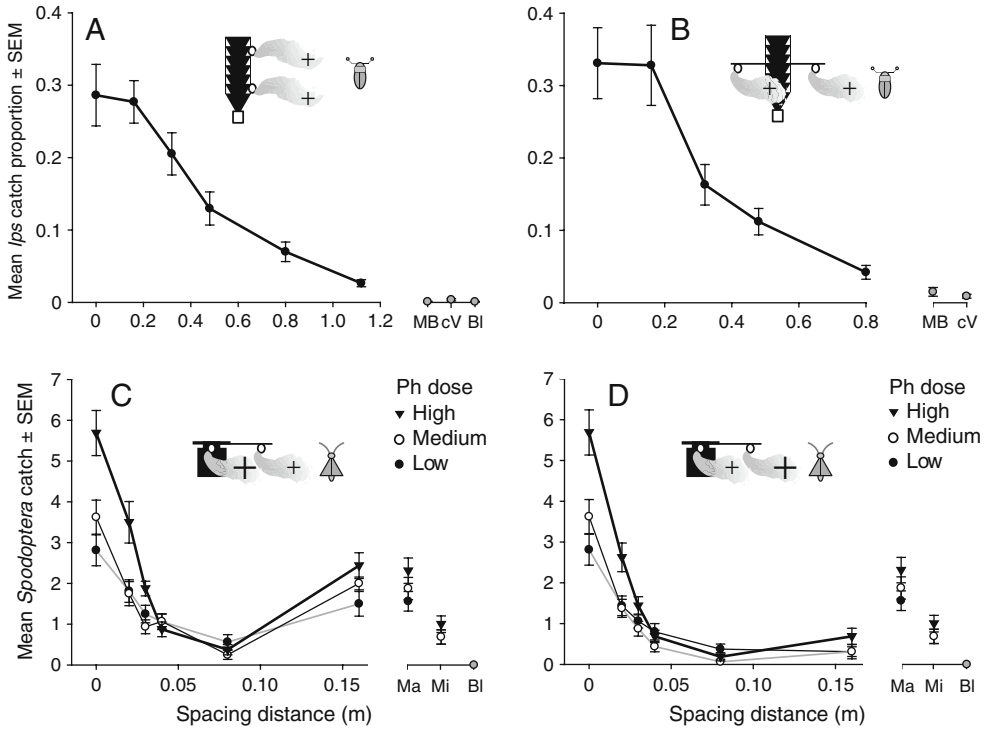


Fig. 3 Trap catches of *Ips typographus* with **a** vertical, and **b** horizontal spacing between pheromone components, *cis*-verbenol (cV) and 2-methyl-3-buten-2-ol (MB). Trap catch was affected by spacing distance (*GzLM*: $\chi^2_5 = 95.8$, $P<0.001$ for vertical and horizontal tests collectively, $N=14$). **c** Trap catches of male *Spodoptera littoralis* with horizontal spacing of pheromone components, with the major component at the center of the trap ($N=16$). Three doses (Low, Medium, High; 5, 50, 500 μg, respectively), based on major component, were tested. *GzLM* indicated that dose ($\chi^2_2 = 10.5$, $P<0.01$) and spacing distance ($\chi^2_5 = 228.9$, $P<0.001$) had effects on catch. **d** Trap catches of male *S. littoralis* with horizontal spacing of pheromone components, with the minor component at the center of the trap ($N=16$). Three doses, as in (**c**), were tested. Dose ($\chi^2_2 = 10.0$, $P<0.01$) and spacing distance ($\chi^2_5 = 289.2$, $P<0.001$) had effects on catch. Right panel in graphs shows response to control treatments. Controls are the same in (**c**) and (**d**). *Ma* major component Z9E11-14Ac; *Mi* minor component Z9E12-14Ac; *Bl* blank

control (IT-REP, low dose, 1 m distance). The anti-attractive effect was similar to the effect of the IT-REP dispensers positioned 2 m (low dose) from the trap ($g \approx -1.7$ for both treatments). The flakes appeared to have a greater suppression of trap catch during the first day in the field, when 22% of the beetles were caught by this treatment (c.f., during the second day, the catch was 32%). However, this tendency was not significant, as shown by the absence of a statistical interaction between treatment and capture day (*ANOVA*: $F_{2,\ 54}=2.1$, $P>0.05$); therefore, data for the 2 days were analyzed together.

Effect Size Comparison Spacing of odor sources had strong effects on both species, but similar effects were obtained at very different spacing distances (Fig. 6a). This comparison highlights the superior sensitivity to small-scale spacing of the moth and the potential long-distance effect of NHV on the beetle. For the moth, the effect of the low dose of antagonist was lost when it was separated only 0.02 m from the pheromone. However, for the beetle, strong effects of eight NHV point sources and of ca 6000 NHV flakes were found at meter-scale spacing (Fig. 6b).

Discussion

Beetle vs. Moth The distances at which odor source spacing affected responses differed greatly between the beetle and the moth, falsifying hypothesis *b*. In both the pheromone component and pheromone/anti-attractant spacing experiments, effects on catches of male moths were apparent at spacing distances of a few centimeters (ca 0.01–0.1 m), whereas for beetles, similar effects on catches were apparent at spacing distances approximately one order of magnitude larger (ca 0.1–1 m). The lack of wind vanes on the moth traps means that maximum separation between plumes only occurred at two wind directions, suggesting that the modulation by spacing for the moth could occur at an even smaller scale than indicated by the results.

At a 16 cm spacing, bubbles from the two generators mixed almost immediately, and produced only one discernible plume, but, even so, catches of moths were affected at even smaller spacing distances. Possibly, the smaller moth trap (height: 22 cm, diam.: 13 cm; compared to Lindgren trap, height: >1 m, diam.: 19 cm) was less affected by turbulence, which affects plume structure and flight

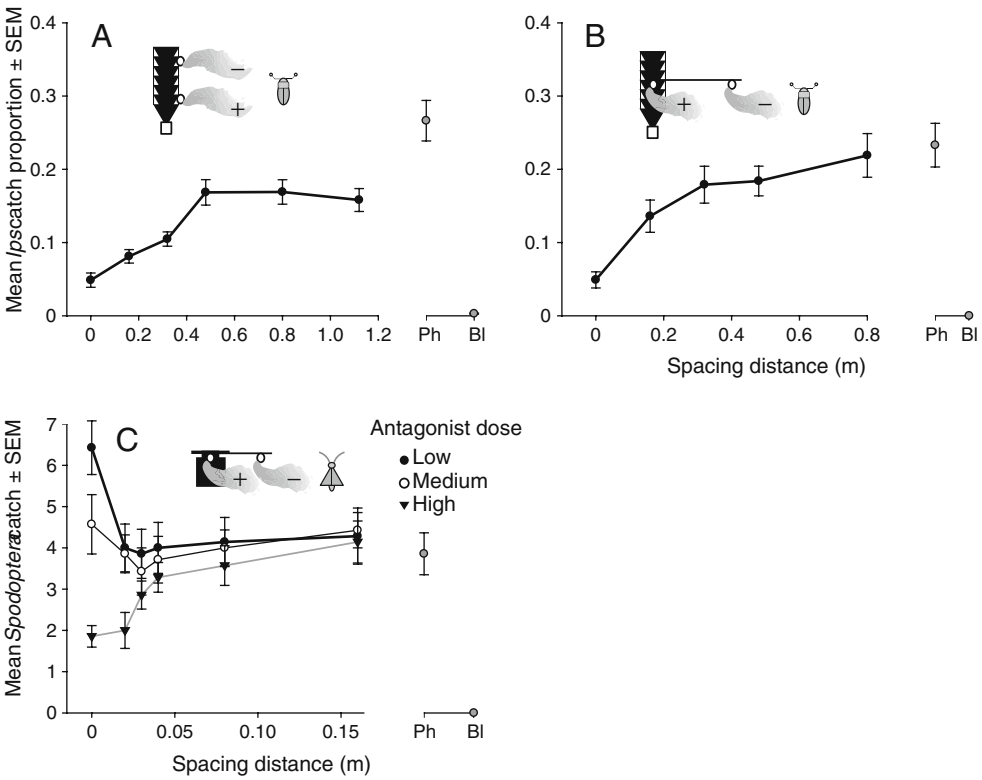


Fig. 4 Trap catches of *Ips typographus* with **a** vertical (N=16), and **b** horizontal (N=14) spacing between pheromone (*cis*-verbenol + 2-methyl-3-buten-2-ol) and anti-attractant (non-host volatile blend) sources. Trap catches were affected by spacing distance (*GzLM*: $\chi^2_5 = 29.8$, $P<0.001$ for vertical and horizontal tests collectively). C) Trap catches of male *Spodoptera littoralis* with horizontal spacing between sex pheromone and three doses (Low, Medium, High; 2.5, 25, 250 µg, respectively) of a pheromone antagonist (N=7). The response to spacing depended on dose, as shown by an interaction between spacing distance and dose ($\chi^2_{10} = 23.7$, $P<0.01$). Right panel in graphs shows response to control treatments. *Ph* pheromone; *Bl* blank

behavior (Willis et al., 1991; Mafra-Neto and Cardé, 1994). Alternatively, mixing of the small scale eddies did not occur, despite the apparent overlap of plumes. It was not possible to measure overlap of plumes from the moth trap, but it appears unlikely that different trap designs would explain the different scales of odor separation that influenced trap catches in the respective species. Possibly, visual cues from the vertical beetle trap, resembling a tree trunk, may have enhanced beetle attraction, especially in the vertical pheromone component spacing test. However, it is difficult to imagine that it could account for the long-distance, anti-attractive effect of NHV. Odor plumes differ between fields and forests (Murlis et al., 2000). However, as the beetle tests were carried out in open areas, it is unlikely that physical environment had a significant influence on plume structure in the different tests.

Plume overlap depended on distance between sources, confirming hypothesis *a*. At 0.5–1 m source spacing, plume

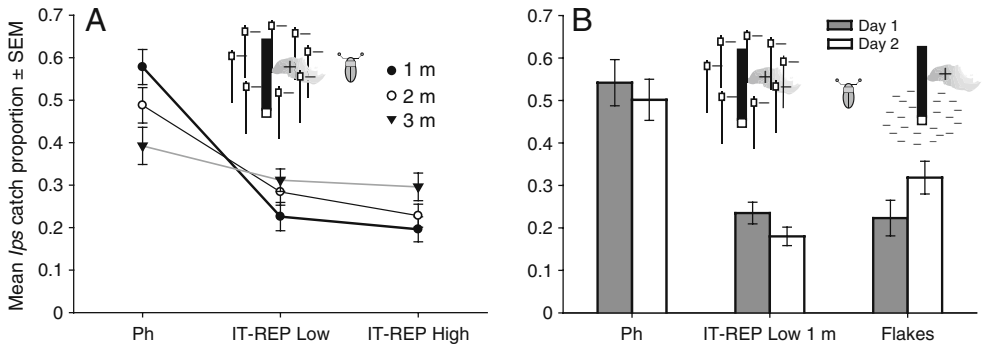


Fig. 5 **a** The effect of surrounding a pheromone trap with eight anti-attractant point sources [IT-REP non-host volatile (*NHV*) dispensers; two doses, low and high, at 1, 2, or 3 m distance from the pheromone] on *Ips typographus* trap catch (N=8–10). *Ph* pheromone control trap. The distribution of beetles among the three treatments was dependent on distance between pheromone and NHV, as shown by the interaction between treatment and distance (factorial *ANOVA*: $F_{4,\ 69}=4.98$, $P<0.001$). **b** The effect of NHV treatment on *I. typographus* trap catch (N=10). Flakes=ca 6000 NHV-treated flakes. *ANOVA* demonstrated a difference among treatments ($F_{2,\ 27}=18.5$, $P<0.001$). Catch in traps surrounded by flakes was lower than that in the pheromone control ($P<0.001$), and not different from that of the IT REP (low dose, dispensers 1 m from trap) negative control ($P>0.05$). Note: as the experiments (1, 2, 3 m distance, and flakes) differed greatly in the total number of beetles caught, we analyzed the effect of NHV only on relative catch

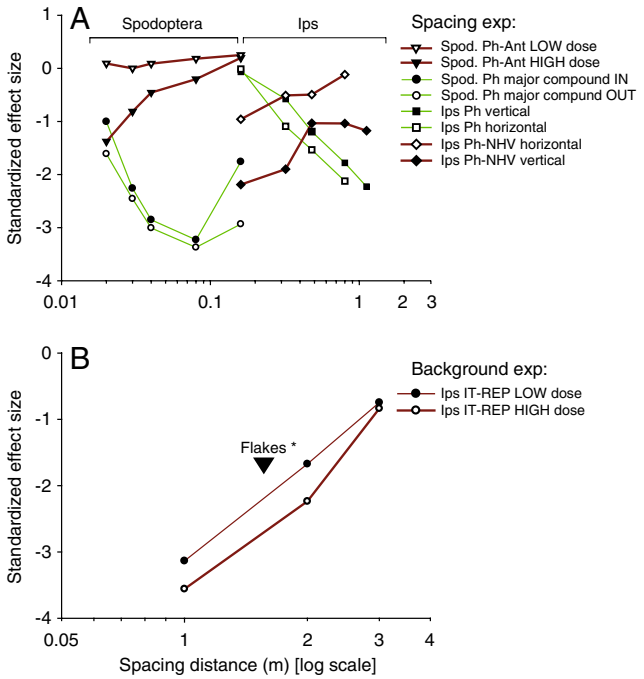


Fig. 6 **a** Effect sizes for the various spacing distances in the *Ips typographus* and *Spodoptera littoralis* spacing experiments (*Hedges'* unbiased *g*). The effect size provides a measure of a biological treatment effect, by scaling the difference between the treatment and control means, with the pooled standard deviation for those means. Effect sizes further from zero than 0.8 are regarded as strong effects. In all experiments, the pheromone bait alone (zero distance between components) was the control. The 0 cm spacing distance in experiments involving anti-attractants is omitted for clarity. **b** Effect sizes in the *Ips* anti-attractant background experiments using IT-REP dispensers at eight positions, or flakes around the trap. * Flakes were evenly distributed on the ground 0–2 m from the trap. Thus, this treatment is "not to scale" on the x-axis. *Ph* pheromone; *Ant* antagonist (*Z*)-9-tetradecenyl acetate; *NHV* non-host volatiles

overlap typically started ca 1–3 m from the trap, suggesting that beetles discern filaments from the two plumes several meters downwind. As indicated by our PID measurements, at 1–2 m from the source, the frequency and concentration of bursts were much lower than when closer to the source, suggesting that filament discrimination in the two insects is performed under different olfactory conditions. However, changes in wind direction may have lowered the frequency of filament encounters by the stationary PID more strongly further downwind. The measured drop in concentration over decimeter-scale downwind distances was sharper than previously found in a wind tunnel (Justus et al., 2002). It is possible that the lower sampling rate of our detector is a reason for this difference. In the field, however, plumes have not been measured at such small downwind distances previously (Murlis and Jones, 1981; Murlis et al., 2000).

The different scale of source spacing distances that affected trap catches in the two species may reflect the actual difference in size of the natural odor sources to which they orient. A male moth orients to a single calling female, whereas bark beetles often orient to large patches, with hundreds of calling individuals. This likely has resulted in different selection pressures on the olfactory system of the two insects (as discussed by Murlis et al., 1992). The male moth pheromone processing system has been selected for locating mates. In contrast, in bark beetles, aggregation pheromone may be used to locate multiple resources, including food, mates, and oviposition sites; therefore, a wider range of selection pressures and tradeoffs may have come to bear on the bark beetle olfactory system. The pheromone processing subsystem in moths, in general, appears more specialized than that of bark beetles. In moths, olfactory receptor neurons (ORNs) for pheromone components and antagonists typically are housed within a special type of sensillum (*trichodea*), different from the sensilla that detect plant odors (Ljungberg et al., 1993). In contrast, *I. typographus* harbors at least one sensillum that contains a pheromone ORN, detecting cV, in addition to an ORN for the plant compound 1,8-cineole (Andersson et al., 2010). In the antennal lobe (AL), axons of moth pheromone ORNs project to glomeruli in an enlarged, and male specific, macroglomerular complex (MGC) (Galizia and Rössler, 2010). Although the ALs of bark beetles are not as well-studied, preliminary observations indicate no apparent MGC in *I. typographus* (Andersson M.N., unpublished).

Mechanisms Variability (difference between maximum and minimum estimates) in plume overlap increased with distance between bubble sources; even for the larger spacings, plumes occasionally overlapped close to the trap. Only when plumes overlap can an insect detect filaments from two plumes coincidently. Therefore, it is possible that insects entered (or avoided, depending on experiment) a trap predominantly when overlap at close range to the sources occurred. We speculate that variation in trap catches may partly reflect variation in the duration of plume overlap close to the trap. Coincident detection of pheromone components improves temporal spiking pattern resolution of projection neurons in moths (Christensen and Hildebrand, 1997), which seems consistent with the behavioral effects of separation between odor components (Fadamiro et al., 1999). The lack of bark beetle response to small-scale spacing may imply that coincidence detection is of less importance for bark beetles.

Pheromone Component Spacing For *S. littoralis*, the major component alone, and the 16 cm spacing between pheromone components (major component in center), captured more males than the 8 cm spacing between components. Possibly, when component sources are separated by 16 cm,

a moth may perceive the two signals as distinct and, therefore, will orient toward the most attractive one. In contrast, at 8 cm the moth may perceive only a single low-quality signal and, therefore, will arrest its movement toward the source. Similar results have been found previously (Linn and Gaston, 1981; Lux et al., 1994). In contrast, the beetle appeared unaffected by 16 cm spacing between pheromone components; some beetles were captured even when the components were separated by >0.5 m. In the western pine beetle, *Dendroctonus brevicomis*, attraction to pheromone was observed when components were separated by as much as 4 m (Byers, 1987).

Pheromone/Anti-attractant Spacing For both the beetle and the moth, the effects on trap catches in response to spacing of pheromone and anti-attractant occurred at separation distances similar to those in response to spacing of pheromone components, thus confirming hypothesis *c*. The bark beetle vertical test indicated that NHV probably influences pheromone attraction over long distances, as the 112 cm spacing distance between pheromone and NHV attracted fewer individuals than the pheromone control. However, no such effect was found in the horizontal test. The fact that soap bubbles sometimes went straight up suggests that convective air movements from the ground in the clearings (possibly pronounced inside the sun-heated black Lindgren trap in the vertical test) may have resulted in mixing of plumes more frequently in the vertical than in the horizontal tests.

The ability of *S. littoralis* to distinguish small separation distances between pheromone and antagonist is similar to that found for other moths (Witzgall and Priesner, 1991; Liu and Haynes, 1992; Fadamiro et al., 1999). Interestingly, Z9-14Ac (our "antagonist") reduced trap capture only at the highest dose, with the lowest dose actually resulting in increased trap capture. As this compound is found in low amounts in the pheromone gland (Nesbitt et al., 1973), this suggests that Z9-14Ac could be a minor component of the sex pheromone, with high doses distorting the natural blend ratio and producing an antagonistic effect. A similar result was found for an antagonist of the moth *Cydia pomonella* (Coracini et al., 2003).

Applications Forest pest insect infestations typically occur less frequently in diverse habitats (Jactel and Brockerhoff, 2007). This may be due, in part, to the presence of non-host volatiles that impede host location (Jactel et al., 2011; Schiebe et al., 2011), as suggested by the "semiochemical diversity hypothesis" (Zhang and Schlyter, 2003). Our two NHV background experiments indicate that attraction to pheromone by bark beetles was reduced by NHV within 2–3 m of the pheromone source. These results are largely consistent with the previously determined "active inhibitory range" (AIR) of NHV, and support our semiochemical diversity hypothesis (Zhang and Schlyter, 2003). It is striking that the NHV background reduced catches of beetles to such a large extent, especially as the pheromone dose used was high, comparable to that from a mass-attacked tree (Schlyter et al., 1987b). However, trap catch was not shut down completely, suggesting that NHV may be more effective for reducing the risk of trees being attacked (Jakuš et al., 2003), rather than counteracting mass-attacks. Indeed, successful attempts to protect standing trees using NHV dispensers attached to every second tree showed an effect over ca 2 m, comparable to the typical distance between trees (Jakuš et al., 2003). In yet another study, groups of 10 standing trees were protected with 20 NHV dispensers, with beetle attacks diverted to trees >15 m away (Schiebe et al., 2011).

Approximately 6000 NHV flakes gave equivalent reduction in trap catch as did the 8 IT-REP dispensers. Thus, the (presumed) more uniform 'cloud' of NHV from the large number of flakes did not 'mask' the pheromone any better than the 8 point sources, thus falsifying hypothesis *d*. Flakes releasing the anti-attractant verbenone protect pine trees from attack by *Dendroctonus* bark beetles (Gillette et al., 2009), but whether they can be used successfully in forest protection against *I. typographus* needs further investigation.

For *S. littoralis*, the effect of separating pheromone components was stronger than expected; the major component alone was more attractive than several treatments with both components spatially separated. This could have implications for pheromone mating disruption. In most, but not all, cases of mating disruption, more complete pheromone blends have provided greater disruption (Minks and Cardé, 1988). This is expected if the dominating mechanism is competition with calling females (Miller et al., 2006). However, if moths orient to spatially separated pheromone components and arrest once plumes no longer overlap, disruption may be increased (Byers, 1987). However, as a note of caution, separate dispensers may be less feasible for large-scale use.

In summary, a comparative approach that uses different insect taxa may allow a more general understanding of insect olfactory-mediated behaviors to be developed. Our results suggest that, at least for the beetle, orientation to attractive sources may be hampered in semiochemically diverse habitats. Additionally, the moth was more sensitive than the beetle to spacing distances between odor sources. However, the source spacing distances that affected trap catches were similar in the two tests (i.e., pheromone source spacing, and pheromone/anti-attractant spacing) for both species. The two NHV background experiments on *I. typographus*, in addition to three other differently designed studies (Jakuš et al., 2003; Zhang and Schlyter, 2003;

Schiebe et al., 2011), indicate that NHV could be used in forest protection to reduce the risk of bark beetle attacks.

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ketones (Raspotnig et al., 2005; Jones et al., 2009). Hitherto known secretions of the Palpatores include acyclic ketones, alcohols, and aldehydes, present in some sclerosomatid Eupnoi (Blum and Edgar, 1971; Meinwald et al., 1971; Jones et al., 1976, 1977; Ekpa et al., 1985), and naphthoquinones reported from one species of phalangiid Eupnoi (Wiemer et al., 1978). Only one study is available on scent gland secretions of the Dyspnoi, demonstrating the presence of naphthoquinones, methoxy-naphthoquinones, and anthraquinones in the scent glands of a nemastomatid species (Raspotnig et al., 2010). However, with respect to the number of species investigated, secretions of the mostly tropical Laniatores appear to be best studied. Laniatorean secretions are chemically distinct from the secretions of cyphophthalmids and palpatoreans, and specific patterns of phenols and benzoquinones have been demonstrated from a growing number of species since the 1950s (e.g., Estable et al., 1955; Eisner et al., 1971, 1977, 2004; Roach et al., 1980; Hara et al., 2005; review by Gnaspini and Hara, 2007; Shear et al., 2010a,b; Pomini et al., 2010; Föttinger et al., 2010). Essentially, nearly all published studies deal with representatives of groups of more-derivative Laniatores. Thus, the current knowledge on laniatorean secretions is strongly biased in favor of the Grassatores. The chemistry of scent gland secretions in the Insidiatores – a second, more basal laniatorean group – is almost unknown, but is crucial for a consistent picture of opilionid scent gland chemistry. Only one example of insidiatorean chemistry has been published so far (Ekpa et al., 1984), revealing a series of completely aberrant components from an American travunioid species, *Sclerobunus robustus*. These compounds include terpenes, such as bornyl esters, camphene, and limonene, and two alkaloids, namely N,N-dimethyl-ß-phenylethylamine and the tobacco alkaloid nicotine.

One important and well recognized taxon within the Insidiatores is the Cladonychiidae (superfamily Travunioidea), including about 15 species in 5 extant genera (a 6th genus was described from Baltic amber by Ubick and Dunlop in 2005). While genera *Cryptomaster*, *Erebomaster*, *Speleomaster*, and *Theromaster* are restricted to the northern United States, an exclusively European clade is represented by eight species of *Holoscotolemon*, occurring from Western Europe to Romania and southwards to Serbia and Montenegro. As a first contribution to scent gland research in European Insidiatores, we here report on the scent gland chemistry of four species of *Holoscotolemon*, covering a representative part of European Cladonychiidae.

Methods and Materials

Collection of Species Specimens of *Holoscotolemon jaqueti* (Corti, 1905), *H. lessiniense* Martens, 1978, *H. oreophilum* Martens, 1978, and *H. unicolor* Roewer, 1915, were collected by hand under stones and litter, and from samples of leaf litter by means of a soil sifter. While *H. jaqueti* is a species with a disjunct South-East European and Carpathian distribution (Hungary, Slovakia, Romania, Ukraine, Bosnia and Hercegovina, Serbia), *H. unicolor* is restricted to the Eastern Alps (Austria, Slovenia, North Eastern Italy), and both *H. lessiniense* and *H. oreophilum* are local endemics of the southwestern Alps (Italy). *Holoscotolemon oreophilum* can be found in a small area in the Sea Alps/Alpes Maritimes and Ligurian Alps in Italy, and *H. lessiniense* inhabits the Monti Lessini close to the Lake Garda in the Southern Alps. The investigated material was collected in Serbia (*H. jaqueti*), Italy (*H. oreophilum, H. lessiniense*), and Austria (*H. unicolor*) (Table 1). For determination of the *Holoscotolemon* species, we used descriptions of Martens (1978). The material is stored in the collections of Ch. Komposch (Graz), G. Raspotnig (Graz), and I. Karaman (Novi Sad).

Extraction and Analysis of Secretions Scent gland secretion was obtained by whole body extraction of individuals in 150 µl of hexane or ethyl acetate for about 30 min as previously described and standardized for other opilionids (Raspotnig et al., 2005, 2010). In total, 21 extracts were prepared, mostly extracts of single individuals (adults), but in some cases also pooled extracts (juveniles), as summarized in Table 1. Aliquots of extracts (2 µl) were subject to gas chromatographic - mass spectrometric (GC-MS) analysis, using a Trace GC2000 coupled to a Voyager MS, both from Thermo (Vienna, Austria). The GC was equipped with a ZB-5MS fused silica capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness, Phenomenex, Germany). Injection was splitless with helium (at 1.5 ml/min) as carrier gas. The column temperature was programmed from 50°C (held for 1 min) to 200°C at 10°C/min, and then to 300°C at 15°C/min. The ion source of the MS and the transfer line were kept at 170°C and 310°C, respectively. Electron impact (EI) spectra were recorded at 70 eV.

Reference Compounds Authentic nicotine (mixture of (*R*)- and (*S*)-nicotine) for comparison of GC-MS data was purchased from Sigma (Vienna, Austria). As a reference for authentic anabaseine, we used anabasine (from Sigma, Vienna, Austria), which also contained a small amount of anabaseine (about 1%).

Quantification of Secretion Components Determination of absolute amounts of nicotine in secretions are based on the integration of peak areas in the chromatograms, and comparisons to a calibration curve established for authentic nicotine.

Table 1 Collection of *Holoscotolemon*-species and preparation of extracts

Species	Location (co-ordinates, altitude)	Date	Extracts no.[a](Individuals)
H. jaqueti	Serbia: Ovčar-Kablar Gorge, above Ovčar Banja (43°53′N, 20°11′E, 375 m)	20 Mai 2009	1754 (1 juv), 1755 (1♀), M430 (1 juv)
H. oreophilum	Italy: Alpi Marittime, Coli de Tende S Limonette (44°09′ N, 7°32′ E, 1,475 m)	29 July 2010	2447 (1 ♂), 2448 (1 ♀)
H. lessiniense	Italy: Mt. Lessini, Monte Pasubio (45°46′ N, 11°07′ E, 760 m)	17 July 2009	M353 (1♀), M354 (1♀), M355 (1♀), M356 (1♀)
	Italy: Mt. Lessini, NNE Verona, S Velo Veronese (45°35′N, 11°04′ E, 920 m)	31 July 2010	2449 (1 ♀)
H. unicolor	Austria: Carinthia, Villach, Eichholzgraben (46°38′ N, 13°50′ E, 590 m)	5 April 2010	1935 (1♀), 1936 (1♂)
	Austria: Styria, Graz, Maria Trost (47° 06′ N, 15° 29′ E, 445 m)	20 April 2010	1973 (1 juv), 1983 (3 juv)
		25 April 2010	1995 (3 juv)
		10 May 2010	2000 (2 juv), 2001 (2 juv)
	Austria: Styria, Aibl (46° 41′ N, 15° 13′ E, 400 m)	27 June 2010	2127 (1 juv)
	Austria: Carinthia, Soboth, road between Krumbach and Rothwein (46°81′ N, 15°07′ E, 1,000 m)	23 June 2010	2243 (1♀), 2244 (1♂), 2245 (1♀)

[a] Refers to intern labelling of extracts

Scanning Electron Microscopy For scanning electron microscopy (SEM), specimens were fixed in Bouin, washed, dehydrated, air-dried, and mounted on aluminum stubs prior to sputter coating with gold (AGAR sputter coater, Gröpl, Tulln, Austria). Micrographs (SEM) were taken with a Philips XL30 ESEM (Philips/FEI, Vienna, Austria) at high vacuum mode and 20 kV accelerating voltage.

Results

Chemical Identification of Extract Components GC-MS analyses of extracts of adult *Holoscotolemon jaqueti*, *H. oreophilum*, and *H. lessiniense* revealed two major (components A and E), and three minor compounds (components B, C, and D) (Fig. 1). All components were identified as pyridine alkaloids as outlined in the following and as summarized in Table 2.

Major Components Component A showed an EI-mass spectrum consistent with that of nicotine [3-(1-methyl-2-pyrrolidinyl)-pyridine], exhibiting a molecular ion (M^+) at a mass charge/ratio (*m/z*) of 162, loss of a hydrogen atom (*m/z* 161), and a base ion at *m/z* 84. The base ion corresponded to loss of the pyridyl moiety ($M^+ - 78$). Further fragments were observed at *m/z* 133 ($M^+ - 29$) and *m/z* 119 ($M^+ - 43$), both of which are reported as characteristic of nicotine, and correspond to losses of C_2H_5- and C_3H_7-radicals from the molecular ion by different processes of rearrangement (Duffield et al., 1965). As a reference for comparison of GC retention, we used a racemic mixture of nicotine (nicotine has an asymmetric carbon atom in position 2 of the pyrrolidine-ring). The specific enantiomers, however, were not separable due to the chromatographic conditions used, and both enantiomers eluted as a single peak at RT= 10.54 min, exactly matching the retention time of component A. Thus, we did not determine the chirality of nicotine from *Holoscotolemon* spp. So far, only the (*S*)-enantiomer is known to occur in nature.

The EI-mass spectrum of the second major component (E) had a molecular ion at *m/z* 160, again exhibiting loss of hydrogen (base ion at *m/z* 159), and losses of 15 (leading to the ion at *m/z* 145), 29 (*m/z* 131), and 56 (*m/z* 104) mass units. The spectrum appeared to be similar, but not fully consistent, with reported spectra of anabaseine (a nicotine-related tobacco nicotinoid) from the literature (e.g., Wheeler et al., 1981). However, a comparison of mass spectral fragmentation and retention times to an authentic sample of anabaseine [3,4,5,6-tetrahydro-2,3′-bipyridine] showed full correspondence.

Minor Components On the basis of mass spectral fragmentation patterns of components B and C, two further nicotinic alkaloids were indicated, both showing mass spectra similar to that of nicotine: Component B appeared

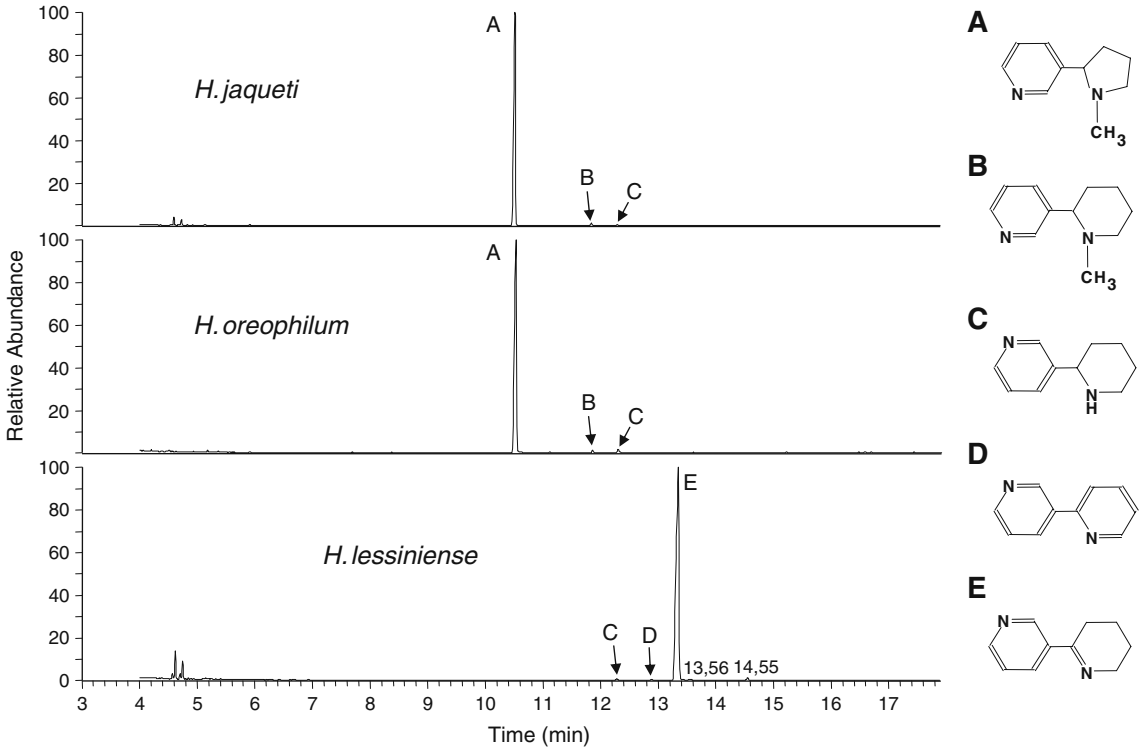


Fig. 1 Chemical profiles of scent gland secretions in three species of *Holoscotolemon*. Compound A [nicotine], compound B [3-(1-methyl-2-piperidinyl)-pyridine], compound C [anabasine], compound D [2,3′-bipyridyl], compound E [anabaseine]. *Compounds at RT=13.56 min and RT=14.55 min were found inconsistently in extracts of *Holoscotolemon lessiniense*, and possibly represent isomeric, not fully characterized pyridines with a molecular weight of *M*=174

to be a higher homolog to nicotine with M^+ at *m/z* 176 and a base ion at *m/z* 98, suggesting a piperidine-structure instead of the pyrrolidine-ring. The compound was tentatively identified as 3-(1-methyl-2-piperidinyl)-pyridine by mass spectral comparisons to literature, and to spectra from the NIST-library. The mass spectral fragmentation of component C differed from that of nicotine mainly in the presence of prominent twin ions at *m/z* 105 and *m/z* 106, being characteristic of anabasine [= 3-(2-piperidinyl)-pyridine]. The compound was identified by comparisons of spectra and retention times with an authentic sample of anabasine. Only the mass spectrum of component D was clearly different from all other spectra, although again showing an intense molecular ion at *m/z* 156 (base ion) along with loss of hydrogen (*m/z* 155). The isotopic M+1-ion (about 12% relative intensity) still indicated the presence of 10 carbon and 2 nitrogen atoms, suggesting a molecular formula of $C_{10}H_8N_2$, and thus corresponding to the fully aromatic homolog of anabasine and anabaseine, 2,3′-bipyridyl [2-(3-pyridinyl) pyridine].

Table 2 Gas chromatographic and mass spectral data to components from extracts of *Holoscotolemon lessiniense*, *H. oreophilum*, and *H. jaqueti*

Peak	Retention time (min)	EI-fragmentation (*m/z*)	Identified as
A	10.54	162 (M^+, 49), 161 (46), 133 (79), 119 (24), 118 (16), 92 (23), 84 (100), 65 (21), 51 (22), 42 (61)	nicotine = (S)-3-(1-methyl-2-pyrrolidinyl)-pyridine
B	11.85	176 (M^+, 12), 175 (7), 147 (7), 133 (9), 119 (24), 98 (100), 42 (28)	3-(1-methyl-2-piperidinyl)-pyridine[a]
C	12.29	162 (M^+, 26), 161 (23), 133 (45), 119 (45), 106 (49), 105 (60), 92 (19), 84 (100), 80 (22), 78 (20), 57 (29), 51 (28), 41 (38)	anabasine = 3-(2-piperidinyl)-pyridine
D	12.89	157 (12), 156 (M^+, 100), 155 (71), 130 (20), 128 (12), 104 (7), 79 (13), 78 (22), 51 (13)	2,3′-bipyridinyl = 2-(3-pyridinyl)-pyridine[a]
E	13.35	161 (12), 160 (M^+, 97), 159 (100), 145 (46), 132 (14), 131 (79), 118 (8), 105 (44), 104 (76), 78 (27), 77 (29), 51 (36), 41 (29)	anabaseine = 3,4,4,6-tetrahydro-2,3′-bipyridine

[a] Tentatively identified on the basis of mass spectral data

Subsequent mass spectral comparisons confirmed a mass spectral fragmentation pattern indistinguishable from spectra of authentic 2,3′-bipyridyl from literature and from the NIST-library.

Extract Profiles Two different extract patterns were clearly distinguished: (1) Nicotine-rich and (2) anabaseine-rich extracts. The extracts of *Holoscotolemon oreophilum* and *H. jaqueti* were dominated by compound A (nicotine: >97% of the secretion, based on calculation of peak areas, see also Table 2 and Fig. 1). Components B (3-(1-methyl-2-piperidinyl)-pyridine) and C (anabasine) were found constantly as accompanying minor or trace components (each about 1% of the secretion). In *H. lessiniense,* anabaseine (compound E) was the major compound in the extracts (>95%, see Table 2 and Fig. 1), accompanied by trace amounts of anabasine and 2,3′-bipyridyl (each about 1–3% of the secretion). In two out of five extracts, two trace components were also found at RT=13.56 min and 14.55 min (each about 1% of the secretion), both showing a pyridine-like pattern and a molecular weight of 174, but both remain unidentified.

It is noteworthy that some individuals did not show any components. In particular, extracts of three specimens (all collected at one locality in Serbia, see Table 1) of *H. jaqueti* were investigated, and two of these (one female, one juvenile) consistently showed the nicotine-rich composition mentioned above. In the extract of the remaining specimen (a juvenile), no components could be detected. For *H. lessiniense*, we analyzed extracts of five individuals (all female), which were from two different collections (see Table 1): Three extracts (two of collection no. 1, one of collection no. 2) exhibited the anabaseine-rich scent gland profile described above. No components could be extracted from two other specimens (from collection no. 1). For the largest species of the genus, *H. oreophilum*, only two individuals (one male, one female) were available to us, both of which discharged large amounts of nicotine. Absolute amounts of secretions were determined for *H. oreophilum,* and were calculated to be about 15 μg nicotine per individual (11.8 μg measured for one specimen, 16 μg for the other specimen).

Thus, only for *H. oreophilum* (nicotine-rich), could male and female extracts be compared, showing no differences. For *H. jaqueti* (nicotine-rich) and *H. lessiniense* (anabaseine-rich), only females and juveniles (subadult females) were available; between subadults and adults, no differences were recognized.

Holoscotolemon unicolor Contrasting the results obtained from *H. jaqueti*, *H. oreophilum*, and *H. lessiniense*, no components were detected in any of the nine extracts from adults of *H. unicolor.* This was surprising since these specimens were from several different collection sites in Austria and were collected at different times of the year (see Table 1). Scent glands in *H. unicolor*, as well as openings (ozopores) appeared to be as well developed as in the other *Holoscotolemon* species (Fig. 2). However, in one extract of an early-instar juvenile, traces of three nitrogen-containing compounds were detected (not shown), whereas extracts of 11 other juveniles did not show any compounds. The trace components in the single juvenile extract were tentatively identified as dimethyl-isoalkylpyrazines by mass spectral comparisons to literature, and to spectra from the NIST-library (compound 1: a dimethyl-isobutylpyrazine, M^+ at *m/z* 164; compounds 2 and 3: isomeric dimethyl-isopentylpyrazines, M^+ at *m/z* 178).

Discussion

Origin of Extract Components We assume that the compounds found in the whole body extracts of *Holoscotolemon* herein investigated are from the scent glands of these species. This view is based on several facts, and on corroborating evidence from previous studies. First, scent glands are the only glandular structures capable of producing such amounts of secretions; they are large and well developed in all species of the genus *Holoscotolemon*. Corroborative evidence for the scent gland origin of extract components comes from extraction-attempts of individuals with obviously depleted gland reservoirs. We assume that we may have examined many “empty” specimens, as indicated by the lack of any compound in the gas chromatograms of extracts. From previous investigations, we know that depletion of gland reservoirs can be a rapid process, mainly depending on external irritation stimuli. Refill of glandular reservoirs is slow; according to Holmberg (1970), this process takes a few days in phalangiids. We do not know anything about the refilling capability in species of *Holoscotolemon*. In our study, the relation of specimens with well-filled gland reservoirs to specimens with depleted reservoirs was about 1:1 for *H. jaqueti* and *H. lessiniense.* Glandular depletion may be explained by irritating stimuli in the course of collection and transport. Absolute amounts of secretion, as shown for nicotine in extracts of *H. oreophilum,* were calculated to be about 15 μg per individual. Such amounts correspond to a volume of 15 nl of nicotine (which is a liquid with a density of 1.01 g/ml at room temperature), and hence to two hypothetical spherical “droplets” of about 130 μm in diameter. Moreover, droplets of that size would be consistent with the expected dimension and filling capacity of glandular reservoirs in *Holoscotolemon*, at least if glandular dimensions in other opilionids of comparable

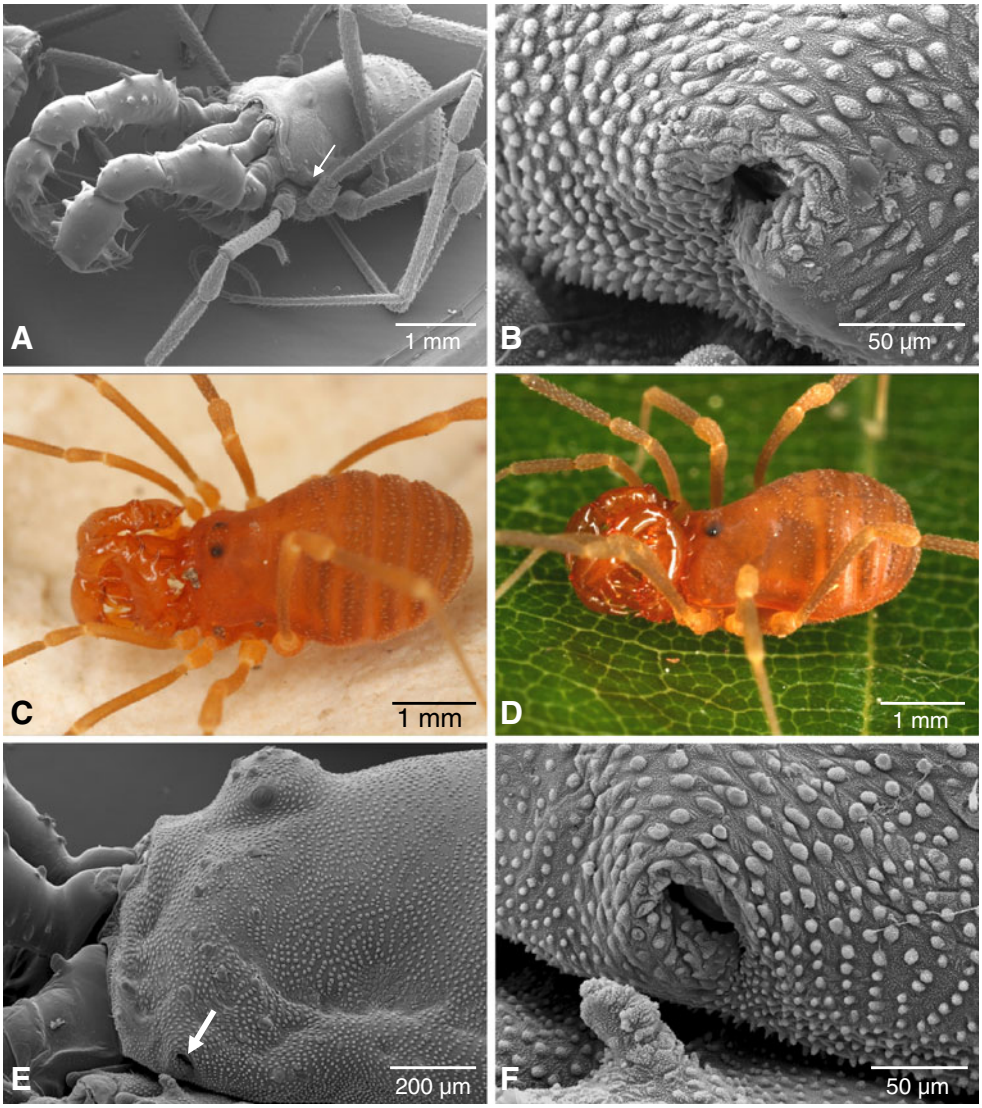


Fig. 2 Position and morphology of ozopores in species of genus *Holoscotolemon*. Arrows point to ozopores that are located near dorsally to coxae II. **a** *Holoscotolemon unicolor,* and **b** detail of ozopore. **c** *Holoscotolemon oreophilum*; **d** *Holoscotolemon lessiniense*; **e** and **f** details of left ozopore in *Holoscotolemon jaqueti*

size are considered (e.g., Juberthie, 1976; Schaider and Raspotnig, 2009).

In general, the whole body extraction-method is widely used and commonly accepted for the assessment of exocrine products in small arthropods that are too tiny to sample or dab their secretions directly from the gland orifices. The method has been standardized for investigations of exocrine glandular products in oribatid and astigmatid mites over many years (Sakata and Norton, 2001; Raspotnig et al., 2001, 2008), and also has been used to assess scent gland exudates of opilionids of different groups (Raspotnig et al., 2005, 2010; Jones et al., 2009; Shear et al. 2010a,b). For instance, in sironids (Cyphophthalmi) direct sampling of secretions from ozopores leads to the same chemical results as working with hexane whole body extracts (Raspotnig et al., 2005).

Nicotine and Anabaseine In general, tobacco alkaloids are unusual components in arthropod exocrine secretions. Nicotine is known only from *Sclerobunus robustus* (a travunioid laniatorean) (www.pherobase.com). Anabaseine, on the other hand, is new for scent gland secretions of Opiliones. However, this compound is a nicotine-derived alkaloid. Nicotine is a defensive neurotoxin of some plants, and anabaseine is a neurotoxin from nemertine worms that use it for paralyzing prey and possibly for deterring predators (Kem, 1971; Kem et al., 1971, 1976). Regarding arthropods, anabaseine-rich secretions appear to be rare. One example, however, is the poison glands of certain myrmicine ants (Wheeler et al., 1981; Leclercq et al., 2001; Co et al., 2003), where anabaseine also may act as a part of the trail pheromone (Attygalle et al., 1998). In certain hymenopterans, anabaseine is found as a by-product in anabasine-rich secretions (Co et al., 2003; Cruz-Lopez et al., 2006). Anabaseine is remarkable for exhibiting striking neurotoxic effects by interacting with nicotinic receptors, and a synthetic anabaseine-derivative has proved to be a

promising test agent to study and cure cognitive function loss associated with several human diseases (Kem et al., 1997, 2006). To our knowledge, *H. lessiniense* is the only arthropod species producing an exocrine secretion almost exclusively based on anabaseine. It is further interesting to note that *H. lessiniense* (anabaseine-rich) and *H. oreophilum* (nicotine-rich), both occurring in the Italian Southern Alps and separated by an air-line distance of just 300 km, use differently composed secretions; this might indicate more specific communicative functions of scent gland secretions in cladonychiid harvestmen, in addition to assumingly defensive roles.

Pyridines and Alkyl-pyrazines Three minor components in the *Holoscotolemon*-secretions were classified as pyridines, chemically close to nicotine and anabaseine. These compounds may represent by-products of the biosynthetic pathways to nicotine and anabaseine, but the possibility of their artificial formation from major compounds in the hot injector of the gas chromatograph cannot be excluded. In fact, small amounts of 2,3′-bipyridyl also could be detected when analyzing an authentic sample of anabasine, calling the scent gland origin of 2,3′-bipyridyl into question. On the other hand, 2,3′-bipyridyl is known to represent a second neurotoxic component in nemertine worms, in addition to anabaseine (Kem et al., 1976), and also occurs in exocrine glands of certain *Aphaenogaster* and *Messor* ants (Attygalle et al., 1998; Co et al., 2003). The alkyl-pyrazines detected in the extract of one single juvenile of *H. unicolor* also are widespread exocrine compounds in several species of ants and wasps (Cavill and Houghton, 1974; Borg-Karlson and Tengö, 1980; Wheeler et al., 1982; Tecle et al., 1987; Brophy, 1989; Jones et al., 1998; Morgan et al., 1999). In some cases, alkyl pyrazines seem to accompany alkaloids of the anabasine-type as minor components in exocrine secretions of some hymenopterans (e.g., Cruz-Lopez et al., 2006). However, our preliminary chemical data for *H. unicolor,* deviating from the chemistry of the other three *Holoscotolemon* species, need further clarification, and we hope to unravel this question in the near future.

Chemosystematics in Genus Holoscotolemon Our study provides an analysis of the scent-gland chemistry of all four Alpine and Carpathian *Holoscotolemon* species. In this context as well as from a zoogeographical and evolutionary point of view, questions concerning the presently inhabited areas of these species are of interest, particularly with respect to the hypothetical regions where they outlasted the last ice-age (Würm). *Holoscotolemon unicolor* is endemic to the Eastern Alps and, following Holdhaus (1954), Thaler (1966, 1976), and Komposch (2009), is a re-wanderer of long distance, which probably survived the last ice age in "massifs de refuge" of the South-Eastern Alps. *Holoscotolemon oreophilum* and *H. lessiniense* are local endemics of the Southern Alps, and should have survived the Würm ice-age in "massifs de refuge" in the areas of their present distribution in the Sea Alps (*H. oreophilum*) and Monti Lessini (*H. lessiniense*), respectively. Martens (1978) found no close relationship between *H. lessiniense* and *H. unicolor*, and believed the former species is closer to *H. oreophilum*. Therefore, it is surprising that the available chemical information points toward a high similarity between the most western (*H. oreophilum*) and the most eastern species (*H. jaqueti*). Thus, tentatively, nicotine may represent a plesiomorphic compound in *Holoscotolemon* (and possibly also in Travunioidea, see below) while *H. lessiniense*, occurring in an area in between, shows a clearly distinct although related chemistry (i.e., anabaseine-rich), possibly representing the derivative status.

Scent Gland Chemistry in Insidiatores The major part of laniatorean scent gland research has been performed on grassatorean Laniatores, and this strong bias may have contributed to the picture of a typical, rather homogenous, phenolic and benzoquinone-rich "laniatorean chemistry". Phenol- and benzoquinone-rich secretions have been identified from representatives of all grassatorean families hitherto investigated, such as many Gonyleptidae (see Gnaspini and Hara, 2007 and references therein; Hara et al., 2005; Machado and Pomini, 2008; Föttinger et al., 2010), Cosmetidae and Manaosbiidae (Eisner et al., 1971, 1977; Roach et al., 1980), Stygnommatidae (Duffield et al., 1981), Stygnopsidae (Pomini et al., 2010; Shear et al., 2010b), and Phalangodidae (Shear et al., 2010a). Even though components other than phenols and benzoquinones (i.e., some accompanying acyclic compounds and alkyl-dihydro-pyrans) have been found in certain Gonyleptidae (Hara et al., 2005; Rocha et al., 2011), nitrogen-containing compounds or even tobacco alkaloids do not seem to occur in the scent glands of the Grassatores. On the other hand, there is growing evidence that insidiatorean secretions – or at least those of superfamily Travunioidea – are based on a completely different chemistry that relies on nitrogen-containing compounds as major secretory constituents. This idea is corroborated by the pioneering study of Ekpa et al. (1984) who found two nitrogen-containing compounds (ß-dimethyl-phenylethylamine and nicotine) in the scent gland secretion of *Sclerobunus robustus*, a travunioid species from both another continent (America) and a second travunioid family (Travuniidae; Kury 2000 onwards). For grouping of "Northern Hemisphere Triaenonychoidea" such as *Sclerobunus* with Travunioidea see Giribet and Kury (2007). Preliminary chemical results from representatives of travuniids in Europe (unpublished) also indicate consistently that tobacco alkaloids constitute major secretory components in species of *Travunia* and *Abasola* (for possible synonymies see Kury

and Mendes 2007) as well as in the so-far systematically unplaced but travunioid *Trojanella* (Karaman, 2005). In this respect, nitrogen-containing compounds: (1) may be widespread or even common in the Travunioidea; and, (2) according to the basal phylogenetic position of Travunioidea within Laniatores, these compounds also may represent the ancestral scent gland equipment of laniatoreans.

Opilionid scent gland secretions generally seem to be a promising pool of group- or even species-specific chemical characters, representing an independent set of data of phylogenetic value, in addition to characters from traditional morphology and molecular genetics. The importance of scent gland chemistry for studies of opilionid phylogeny has been emphasized previously (Roach et al., 1980; Duffield et al., 1981), and more recent papers have added to this topic (Raspotnig et al., 2005, 2010; Jones et al., 2009). So far, an evolutionary line may be traced from the naphthoquinone- and acyclic ketone-rich secretions of the Cyphophthalmi to the similarly structured secretions in eupnoid and dyspnoid Palpatores (see introduction), but any evolutionary link to the chemically distinct secretions of Laniatores is still missing. Additionally, within the Laniatores, the "chemical gap" between the alkaloid-rich secretions of the Travunioidea on the one hand and the generally phenol- and benzoquinone-rich chemistry in the Grassatores on the other hand is not yet well explained. In this regard, information on the unknown scent gland chemistry of the "true" Triaenonychoidea (sensu Kury, 2000 onwards) would be important, as this mainly Southern Hemisphere-distributed group of Insidiatores (see Pinto-da-Rocha and Giribet, 2007) may represent the missing link in the chemosystematic puzzle of harvestmen.

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fish on large outdoor racks. The export of dried stockfish from Norway exceeds 70 million € each year, and the majority of this export is dried Atlantic cod (*Gadus morhua*; Linnaeus, 1758), produced in the Lofoten archipelago. In a typical year, the Norwegian stockfish industry experiences revenue losses of 5–10% resulting from damage to fish from blowfly activity, with local quality downgrading and discarding of fish possibly amplifying losses to 30–40% (values given by the Norwegian Stockfish Association). Stockfish production is confined to a 3–4 month period in the early spring. The only insect species that causes damage to stockfish production is the saprophagous *C. vicina* (Aak et al., 2010b). Despite more than 1,000 years of commercial stockfish production (Sturlason, 1226), few efficient methods for control of this pest are available. Chemical treatment of fish, protective nets, and indoor drying are considered undesirable, because of human health concerns, operational difficulties, and excessive cost.

Mass trapping as a potential control method for *C. vicina* in stockfish production was explored in an earlier investigation, in which dimethyl trisulphide, which attracts *C. vicina* (Stensmyr et al., 2002), was applied to yellow sticky traps and funnel traps (Aak et al., 2010b). In that study, the sticky traps were efficient at catching blowflies during the early phase of stockfish production (February until late April), whereas the funnel traps increased in efficiency toward the end of production (April until the summer). The funnel traps showed high *C. vicina* specificity, and more than 90% of the blowflies caught were female. Overall, in that preliminary study, we estimated that some 5–30% of the flies causing damage were caught, indicating that mass trapping has the potential to reduce blowfly damage in stockfish production.

In the present study, we conducted a 4-year mass-trapping experiment for control of blowflies in stockfish production areas in Lofoten, Norway. Yellow sticky traps were used specifically during the production phase, whereas target-specific funnel traps, with odor bait, were used over the entire season. Damage to stockfish in treatment vs. control areas was used as an indicator of success. Blowfly population monitoring with white sticky traps also was carried out in the mass-trapping areas, so as to establish a connection between blowfly activity and damage, as well as to evaluate the long-term effects of trapping on local blowfly populations.

Methods and Materials

Study Area The Lofoten archipelago is located immediately north of the Arctic Circle and reaches approximately 200 km southwest into the Gulf Stream in the Norwegian Sea. The climate is oceanic, and the islands contain a mix of middle boreal, north boreal, and alpine habitats. Normal temperature (Vestvågøy, 1961–1990) for January is -0.4°C, April 2.3°C, July 12.0°C, and October 5.8°C (Norwegian Meteorological Institute—data base—www.met.no). The mass-trapping experiment was carried out at Gimsøy (68°21′N, 14°05′E), Henningsvær (68°09′N, 14°12′E), Ballstad (68°05′N, 13°33′E), Sund (68°01′N, 13°13′E), and Sørvågen (67°53′N, 13°01′E), located in the central and outer part of the archipelago (Fig. 1).

Experimental Design Five pairs of similar stockfish production areas were selected in cooperation with the Norwegian Stockfish Association (Fig. 1). The experiment was carried out at the main locations for stockfish production in Lofoten. The production sites in each pair were located geographically close to each other, with one site being used for mass trapping and the other as a control. The maximum and minimum distance between pairs of control and treatment was 9 and 1.5 km, respectively. The first year of mass trapping was 2006, and the experiment was continued for 4 consecutive years. Trapping began in the 9th week of each year, which corresponded to the start of drying. Traps were emptied every 4th week: until October (eight trapping periods) in 2006, and until November (nine trapping periods) in the subsequent 3 years. No trapping took place from December–February.

After drying, each stockfish was inspected by professional quality controllers, and the percent-damaged stockfish in the treatment and control areas calculated. Damage levels are influenced by time of hanging and sun exposure (Aak et al., 2010b). To avoid confounding effects of these two elements, an additional test was conducted in the final year. Three hundred fish from each treatment and control area were selected to ensure that drying had begun in the same week, in the control-treatment pairs, and that all fish had received approximately the same level of sun exposure. The 3,000 fish from the 10 sites were inspected by one professional quality controller to avoid subjective differences in evaluation. The origin of each batch was unknown to the quality controller.

The mass-trapping effort in each of the treatment areas was balanced according to the production size (Table 1), but always incorporated at least 10 odor-emitting funnel traps and 30 yellow sticky traps at each site. The number of traps was constant across all years of experimentation.

Yellow Sticky Traps Yellow plastic sticky boards (Komadur ES, highly shock resistant PVC, 330 mm×1,000 mm×2 mm; Vink Plast Kolbotn, Norway) were coated with 85 g Tanglefoot insect trap coating (The Tanglefoot Company, MI, USA). The traps were evenly distributed on sun-exposed sides of drying racks. Flies caught in the traps

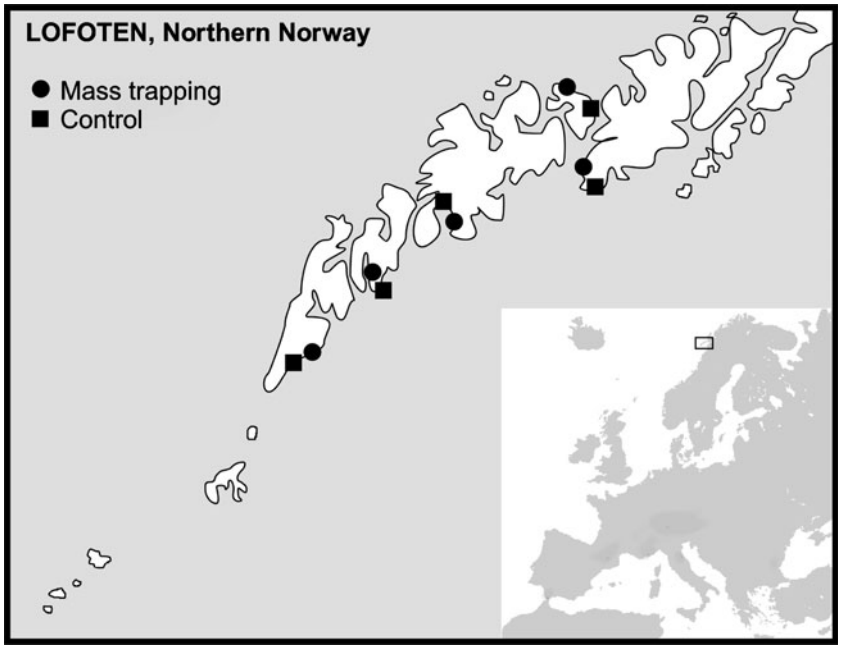


Fig. 1 Location of the 10 stockfish production sites included in the mass-trapping experiment in Lofoten, Norway. Circles represent mass-trapping areas, and squares represent control areas. From north to south the pairs represent stockfish production at Gimsøy, Henningsvær, Ballstad, Sund, and Sørvågen

were identified as *Calliphora* spp. in the field and counted every 4th week, from the start of the drying period until trap stickiness was reduced to such an extent that no additional flies were caught (after 16 week of trapping). To avoid large catches of non-target species, the traps were not replaced or reactivated.

Odor-Emitting Funnel Traps Advantage funnel traps (J.F. Oakes, USA), modified with mesh to reduce the opening of the entry funnel, were used with synthetic lures. The dimethyl trisulphide lure (25%, in ethanol) was replaced in 2007 by a three-component lure (25%, in ethanol), consisting of equal amounts of dimethyl trisulphide (Aldrich Chemical Co, MO, USA, 98% purity), *o*-cresol (Fluka Co, MO, USA, 99% purity), and mercapto-ethanol (Fluka Co, MO, USA, 99.5% purity). The three-component lure was developed by the authors during the mass-trapping work, and was used because it yielded higher catches of blowflies than did dimethyl trisulphide alone (Aak et al., 2010a). One milliliter of lure was placed inside the traps in a 2 ml polystyrene microtube. Traps were evenly distributed in the production area, but always hung at the border of drying racks, and were emptied every 4th week and reactivated with bait. Captured flies were frozen and transported back to the laboratory for counting and identification.

Population Monitoring To monitor blowfly populations in the production areas, we used a small number of white sticky traps made from bucket lids (*Ø*=27 cm) that were coated with 20 g Tanglefoot. These lids were used because they were cheap and practical for field application, and white is as efficient as yellow for catching *Calliphora* spp in Lofoten (Aak, 2010). At each of the 5 sites, 10 traps

Table 1 Location, production volume, trap numbers, and economic effects of blowfly damage at the production sites included in the mass-trapping experiment performed in Lofoten, Norway

Location:	A Gimsøy 68°21′N, 14°05′E	B Henningsvær 68°09′N, 14°12′E	C Ballstad 68°05′N, 13°33′E	D Sund 68°01′N, 13°13′E	E Sørvågen 67°53′N, 13°01′E
Production (tons of dried fish):	70–80	25–30	40–50	80–120	40–60
Number of monitoring traps :	10	10	10	10	10
Number of yellow sticky traps:	40	30	40	50	30
Number of funnel traps:	15	10	14	18	10
Yearly trapping cost:	1,150 €	1,000 €	1,120 €	1,250 €	1,000 €
Average yearly loss:	25,000 €	7,500 €	12,500€	50,000 €	65,000 €

The production size and annual loss are based on numbers reported by the producers, whereas the cost of trapping is based on trap prices and the cost of preparing, mounting, and maintaining traps

were mounted vertically, 1.5 m above ground, on sun-exposed, south-facing, sides of drying racks. The monitoring traps were replaced at the end of each 4-wk trapping period. Continuous monitoring in control areas was not performed. Instead, a point measurement was obtained in these areas (10 traps at each control site, mounted in sun-exposed areas) during the 3 rd trapping period, when fly activity was expected to be maximal (Aak et al., 2010b). Because of administrative and practical difficulties, this point measurement was obtained only in the 4th year of the experiment. The numbers of flies in the treatment-control pairs were compared, and female *C. vicina* were dissected for evaluation of egg developmental state, according to three ordinal categories: 1) white-to-translucent follicles delineating a circular structure with no developed eggs; 2) medium developed, circular-to-ellipsoid eggs with yolk; and 3) fully developed elongate eggs.

Statistical Methods For comparisons between two groups, we used *t*-tests or paired *t*-tests, and for multiple comparisons we used ANOVA. In cases in which tests for normality failed, and it was not possible to normalize data through transformation, we used Wilcoxon Signed-Rank tests. Ordinal logistic regression was used in tests with the categorized data on egg development. In the pair-wise comparison of damage levels to fish, two points had to be removed from the data set, because production was lacking in the control area. One additional measurement was excluded because of extremely late hanging dates resulting in almost 100% damage; this site also was removed from correlation analyses, together with one datum point from 2008, in which the producer failed to kill larvae in the storage room, resulting in release of an unknown, but very high, number of flies at the stockfish production site.

Results

Blowfly Trapping Approximately 306,000 *C. vicina* were caught during the study. Of these, 10.6% were caught with the monitoring traps, 21.1% with yellow sticky traps, and 68.3% with odor-emitting funnel traps. The monitoring traps caught similar numbers of males and females, while the funnel traps caught five times more females than males. The sex ratio was not investigated in the yellow sticky traps.

The white sticky traps used for population monitoring of *C. vicina* showed two distinct activity peaks in all 4 years (Fig. 2). One small peak appeared during production, whereas the second, and most numerous, peak appeared 1 month after production. The catch in the monitoring traps showed seasonal trends similar to the yellow sticky traps during the production period, but diverged from the funnel traps after the production period with a more abrupt drop in fly density following the trapping period in June/August (Fig. 3a, b). There was also a large discrepancy between monitoring traps and odor-emitting traps in May/June when the fish was removed from the drying areas. At this time, odor-emitting traps maintained a high catch while monitoring traps showed a drop. The difference in total catch per production site, between yellow sticky traps and funnel traps, varied according to trapping period (Fig. 3a). The funnel traps had lower catches in the first (*t-test*; t=2.38, N=5, P=0.044) and second (*t-test*; t=6.23, N=5, P<0.001) trapping periods, compared to the yellow sticky traps. The total catch was similar for both trap types in the third period, whereas funnel traps caught more flies than yellow sticky traps in the fourth period (*t-test*; t=8.32, N=5, P<0.001), and continued to catch flies until November, when trapping was terminated.

Effect of Trapping Pair-wise comparisons between treatment and control plots showed that damage caused by blowflies was lower in the mass trapping areas (*Wilcoxon Signed-Rank*; T=27.0, N=17 pairs, P=0.046). The average relative damage reduction varied from 8–31% across experimental years (Table 2). In the final year, when care was taken to ensure similar times of hanging and sun exposure levels, a damage reduction of 63% was found (*Wilcoxon Signed-Rank*; T=7.5, N=5 pairs, P=0.031; Fig. 4).

The total number of flies caught in the monitoring traps each year did not change over the study (Fig. 2; *ANOVA*; $F=0.75_{3,16}$, P=0.54). Comparing control and treatment sites in the third trapping period in 2009, no differences with respect to total number of flies (*paired t-test*; t=0.351, N=5 pairs, P=0.743), number of males (paired *t*-test; t=0.375, N=5 pairs, P=0.727), or number of females (*paired t-test*; t=0.226, N=5 pairs, P=0.832) were observed. However, flies in the control areas had more developed eggs than flies in mass trapping areas (*Ordinal logistic regression*; *Chi-sq*=23.2, *df*=1, P<0.001), with average category scores of 2.6±0.07 (N=98) and 2.2±0.05 (N=95), respectively. There was also a significant difference between the five localities (*Chi-sq*=12.5, *df*=4, P=0.014) but, because there was no significant interaction between treatment and location, the effect from trapping on egg development was present at all sites.

Damage and Blowfly Activity The size of the large summer cohort of *C. vicina* adults (average of the 5th and 6th trapping periods; the two trapping periods following the stockfish production, Fig. 2) was positively correlated with blowfly damage during the production period (r^2=0.58, N=18, P<0.001).

To investigate the correlation between blowfly activity during production and damage to stockfish, we performed linear regressions with the number of blowflies caught in the monitoring traps in each of the first four trapping periods

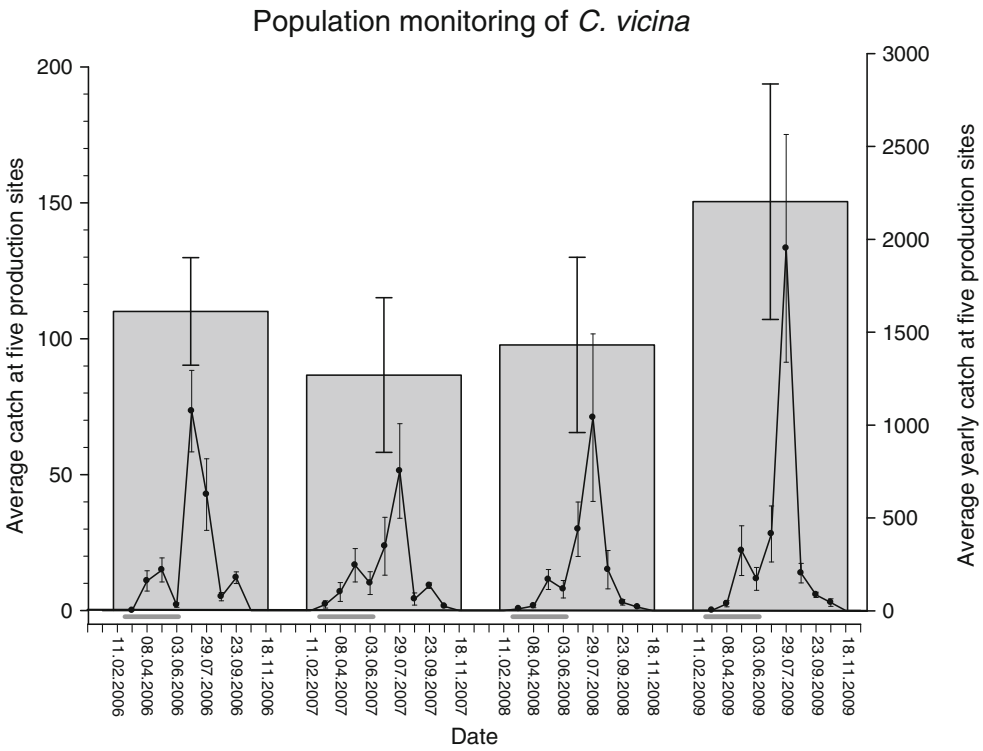


Fig. 2 Adult *Calliphora vicina* caught in white monitoring traps during the 4 years of mass trapping (2006–2009). The grey bars represent the average (± SE) yearly catch at the five production sites (*right y-axis*), and the line represents the average (± SE) catch at the five production sites in each trapping period (*left y-axis*). The stockfish production period is indicated by the thick grey line

(February to June in Fig. 2) as the predictor variable, and the percent damage the same year as the response variable. In the first trapping period, fly activity was low and damage to fish was independent of fly density (r^2=0.01, N=19, P=0.734). In the second period, fly numbers influenced damage slightly (r^2=0.20, N=19, P=0.058), whereas, in the third period, increasing fly activity strongly influenced damage (r^2=0.66, N=19, P<0.001). The connection between the number of flies and damage was weak in the fourth trapping period (r^2= 0.16, N=19, P=0.087), when the number of flies in the monitoring traps decreased.

Discussion

Control programs that target sheep-striking blowflies by using natural odor sources or synthetic lures have shown promise with regard to damage reduction and blowfly population suppression (Anderson et al., 1990; Smith and Wall, 1998; Broughan and Wall, 2006; Urech et al., 2009). Entry traps, or sticky surfaces that combine olfactory and visual stimuli, commonly are used to trap blowflies (Broce et al., 1977; Jiron, 1984; Anderson et al., 1990; Green and Warnes, 1992; Wall et al., 1992; Wall and Smith, 1996; Hall et al., 2003). Synthetic lures also have been developed for screwworm flies (Mackley and Brown, 1984; Horton et al., 1999; Morris, 2005; Urech et al., 2009). In this study, we demonstrated that mass trapping of *C. vicina* blowflies is an economically viable control method in stockfish production.

Our mass-trapping study demonstrated a reduction in damage of stockfish by *C. vicina* of 8–31% per year. The pairs of control and mass trapping sites were kept as similar as possible but, after adjusting for drying time and sun exposure, both of which contribute to damage (Sømme and Gjessing, 1963; Aak et al., 2010b), an even larger reduction (63%) in damage was obtained. This figure more likely reflects the potential impact of mass trapping *C. vicina* in this production system. One might expect that reduction in damage to stockfish should lead to reduced local population densities over time. However, this was not observed. Even when we achieved significant levels of damage reduction, relative to the control, we did not observe any changes in fly numbers over the duration of the trial (i.e., from the first to the last year). The blowfly population density, as measured by catches in the monitoring traps, was determined mainly by flies appearing after production, and these numbers correlated with damage to stockfish 2 months prior. Because the variation in mean damage (3.5–11.2%) among years was much larger than the mean difference in damage (0.6–3.0%) between control and treatment sites, any long-term population suppression effects would have been masked by the large among-year variation. As constant population monitoring was not performed in the control areas, a difference in fly density in mass trapping areas, relative to control areas, could not be investigated.

Fish only are susceptible to fly oviposition during the first 4–6 weeks of drying (Sømme and Gjessing, 1963; Aak et al., 2010b). As the connection between fly activity and damage increases from the second to third trapping periods,

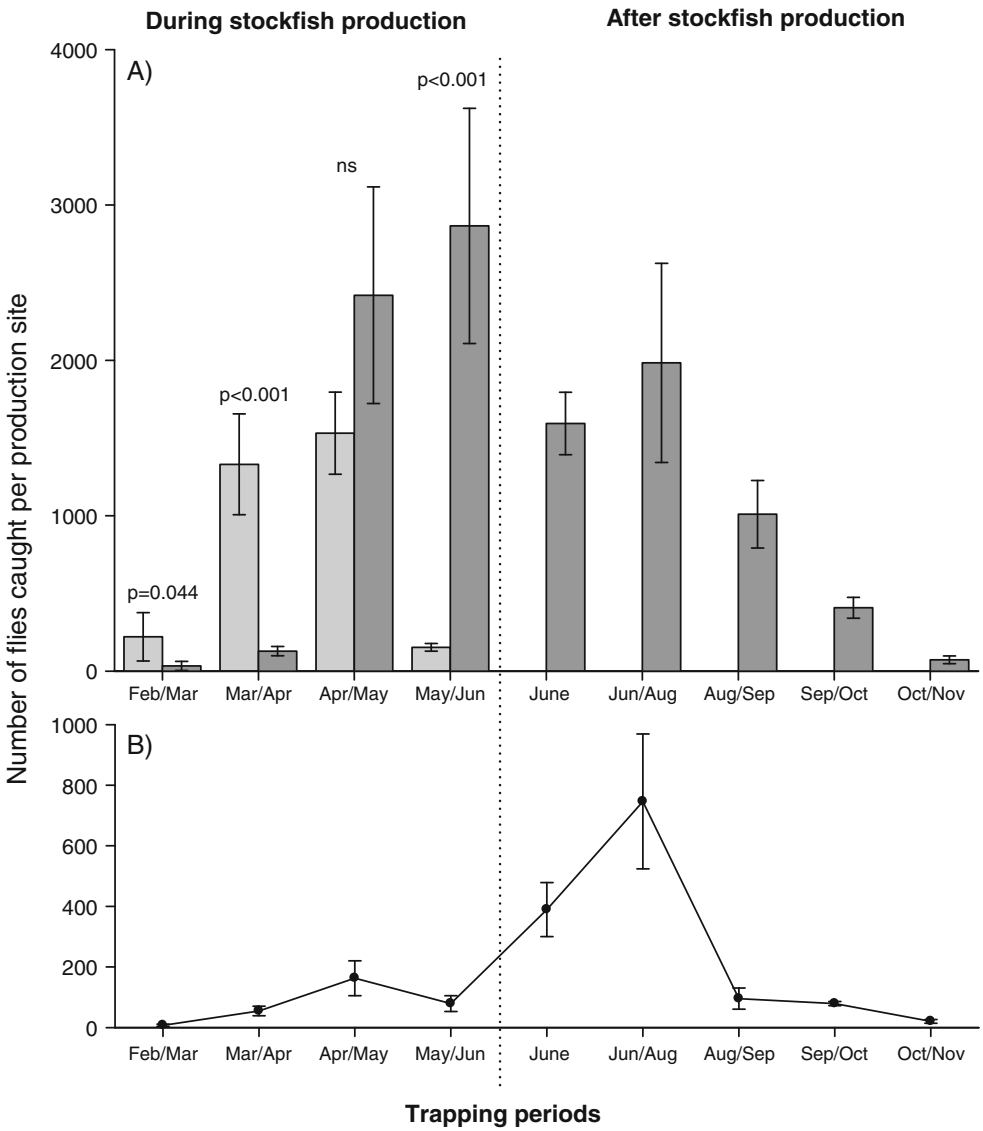


Fig. 3 Average catch (± SE) of *Calliphora vicina* per trapping period at five production sites, from 2006–2009. The dotted vertical line indicates the end of stockfish production. **a** Yellow sticky traps (*light grey bars*) and funnel traps (*dark grey bars*) baited with a synthetic lure consisting of dimethyl trisulphide, *o*-cresol and mercaptoethanol. Significant differences between the trap types in a trapping period are indicated by corresponding *P*-values. **b** White monitoring traps

it is likely that most of the infestation actually occurred in the transition between these periods. At this time, blowfly activity is high, and the fish is not yet dry enough to preclude larval infestation. Most egg-laying blowflies present at this time are likely to belong to a cohort with a univoltine lifecycle, originating from larvae that developed on stockfish in the previous production year (Aak et al., 2011). Blowflies also may breed in natural resources to produce autumn offspring, which spend the winter as larvae and pupae (Aak et al., 2011). The appearance of a second cohort of adults, emerging to co-exist with the parental generation in early spring, may mask a reduction of fly

Table 2 Percent damage to stockfish per year caused by *Calliphora vicina* in the mass-trapping experiment

	Control area	Mass trapping area	Range	Damage reduction
2006	7.6±2.7	7.0±2.3	4.0–13.6	8%
2007	7.1±2.5	5.8±2.2	0.3–11.7	18%
2008	3.5±1.6	2.4±0.9	0.0–7.5	31%
2009	11.2±3.2	8.2±3.2	1.1–17.0	27%

Data were obtained from five pairs of mass-trapping and control sites

The relative damage reduction was calculated from the average difference between the two treatments each year

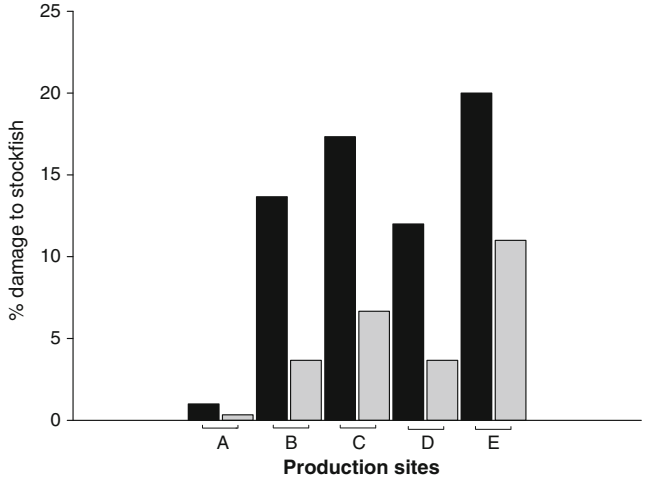


Fig. 4 Percent damage to 3,000 stockfish from five pairs (**a**–**e**) of control (*black bars*) and mass-trapping sites (*grey bars*) in 2009. Fish were selected to ensure that drying had begun in the same week for each control-treatment pair and that all fish received approximately the same sun exposure

numbers, and could also explain our inability to detect differences in blowfly numbers or sex ratios between the control and treatment areas.

The funnel traps were effective from the third trapping period, and caught flies throughout summer to remove egg-laying females from the population. These traps also maintained a higher catch in late summer and autumn, as compared to the more passive monitoring traps. This indicates a selective removal of reproductive females and may contribute to a reduction in the number of over-wintering flies capable of causing damage the following spring. It is possible that the measured reduction in egg developmental state in the mass trapping areas, as compared to the control areas, could be explained by this year-long selective trapping. However, if a mix of cohorts were to occur in the third trapping period, selective trapping of the elder cohort during production also would result in decreases in egg developmental status of the females in the mass trapping areas, relative to the control.

Even though the overall catch was largest in the female-specific funnel traps, the sticky traps caught most flies during the early drying periods, when flies were likely to commence oviposition on the drying fish. Because of this, and the large number of flies caught, the relative contribution of the sticky traps probably was important for the damage reduction observed in this experiment. Thus, such traps should be included in future mass-trapping programs. In a preliminary study, yellow sticky traps were baited with dimethyl trisulphide to increase blowfly catch (Aak et al., 2010b). However, addition of the lure did not appear to change the early efficiency of these traps compared to the lack of lure used in the present study. Most likely, low volatility of the lure at low ambient temperatures, or odor competition from fresh fish, rendered the lure ineffective during the first trapping periods.

Attracting relatively high numbers of adult females, extensive trapping prior to damage, and a pest species with a predominantly univoltine lifecycle, are important factors that contribute to the success of mass trapping programs. These factors were present in our study system, and a successful reduction in blowfly damage in stockfish production was achieved. El-Sayed et al. (2006) reviewed mass trapping as an insect control method, and defined some additional important elements contributing to success: 1) high levels of attraction, capable of competing with natural odor sources; 2) efficient traps at proper densities; 3) isolated insect populations with low immigration rates; and 4) appropriate biology of the pest species; specialized species being considered more advantageous. In our mass-trapping program, we (at least partially) avoided competition with natural odors by trapping flies before stockfish production. The traps also caught flies during production, indicating an ability of traps to compete with fish drying on the racks. We did not investigate the effect of trap density as such, but our high catches of females indicate that the density we used was relatively efficient at removing large numbers of relevant (i.e., adult females) individuals. *Calliphora vicina* can be regarded as a generalist saprophage, having larval development in substrates ranging from garbage to living animals (Ferrar, 1987; Rognes, 1991). Nevertheless, natural larval breeding sites are scarce, and *C. vicina* has a strong, specialist-like response to cues from decaying protein resources (Stensmyr et al., 2002; Angioy et al., 2004; Aak et al., 2010a). No information exists regarding the degree of population isolation, or of immigration/emigration rates at the stockfish production sites, but as these specialists are considered strong flyers, their range of activity may be large.

Mass trapping of *C. vicina* is effective and its use may help to increase profitability for stockfish producers. The yearly trapping costs at the five production sites ranged from 1,000–1,250 €, which is relatively small compared to the estimated loss inflicted by the flies, which is in the range of 7,500–65,000 €. The Norwegian Stockfish Association has set the acceptable level of damage to 3%, which we only achieved in some production areas and, as an average over all mass trapping sites, only in 2008. This control method could be augmented by other non-chemical methods, such as regular collecting and killing of blowfly larvae leaving the fish. Moreover, its efficacy could be enhanced by optimizing the number and relative amounts of the two trap types. Wind tunnel attraction of blowflies to the three-component blend, used in the present study, was 64%, while attraction to authentic dead fish odor was 88% (Aak et al., 2010a). Thus, identification of more behaviorally active compounds or adjustments of blend ratios could further enhance trapping efficacy.

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ambrosia beetles in the tribe Xyleborini, there is extensive inbreeding and haplodiploid sex determination (Rabaglia, 2002). Males of *X. glabratus* are haploid and flightless, and mate with sibling or parental females (consanguineous polygyny) within the brood galleries. Diploid females emerge from their natal trees and engage in brief dispersal flights to locate and colonize new hosts.

Although not considered a pest in Asia, females of *X. glabratus* are primary colonizers of hosts in the U.S., capable of attacking healthy unstressed trees. During gallery excavation, females introduce spores of several symbiotic fungi (Harrington et al., 2010), including the pathogenic *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva (Harrington et al., 2008), carried in mycangial pouches located at the base of the mandibles (Fraedrich et al., 2008). The resultant fungal growth (ambrosia) provides food for larvae and adults within the gallery, but the fungus also invades the host vascular system. Similar to the situation with oak wilt (Jacobi and MacDonald, 1980), it is thought that laurel trees infected with *R. lauricola* respond by forming walls within the xylem vessels (parenchymal tyloses) and by secreting gums and resins that block water transport. This results in systemic wilt and ultimately tree death, which can occur in as little as 6 weeks (Mayfield et al., 2008).

Primary host trees in the southeastern U.S. are native *Persea* species, including redbay [*Persea borbonia* (L.) Spreng.], swampbay [*P. palustris* (Raf.) Sarg.], and silkbay (*P. humilis* Nash), but avocado (*P. americana* Mill.) also has been confirmed as a susceptible host (Mayfield et al., 2008). Additional U.S. hosts include sassafras [*Sassafras albidum* (Nuttall) Nees], camphor tree [*Cinnamomum camphora* (L.) J. Presl], spicebush [*Lindera benzoin* (L.) Blume], pondberry [*Lindera melissifolia* (Walter) Blume], and pondspice [*Litsea aestivalis* (L.) Fernald] (Fraedrich et al., 2008), but other woody lauraceous species still need to be evaluated. In Florida, the widespread distribution of hosts, particularly redbay and swampbay, has facilitated a rapid southward spread of the pest complex since its initial detection in the state in 2005. This has been exacerbated by human transport of infested material, particularly firewood (FDACS-DPI, 2010). In February 2011, laurel wilt and breeding populations of *X. glabratus* were detected in swampbay trees in Miami-Dade County (FDACS, 2011), approximately 14.5 km north of commercial avocado groves.

Avocado production in Florida is worth $14 million annually (USDA-NASS, 2011), and replacement costs of commercial and backyard avocado trees in south Florida have been estimated at $429 million (Evans and Crane, 2008). California produces 60% of the U.S. market, with annual production worth $415 million (USDA-NASS, 2011). Mexico is the largest producer worldwide, with 'Hass' production for the 2010–2011 market year forecast to reach 1.24 million metric tons (USDA-FAS, 2010), of which one third is exported, valued at $645 million (UN, 2010). Due to the serious economic threat posed by *X. glabratus*, there is a critical need for effective attractants to detect, monitor, and control the spread of this invasive pest. This is particularly important for early detection at ports of entry in southern California, Mexico, and avocado production areas in South America. Preliminary research has provided no evidence of an aggregation pheromone and no strong attraction to its fungal symbiont, to its frass, or to ethanol (a standard attractant for ambrosia beetles), suggesting that host tree volatiles are the primary semiochemicals utilized by dispersing females (Hanula et al., 2008). Additional studies have identified manuka oil and phoebe oil [essential oil extracts from *Leptospermum scoparium* Forst. & Forst. (Myrtaceae) and *Phoebe porosa* Mez. (Lauraceae), respectively] as attractive baits for field monitoring of *X. glabratus* in South Carolina (Hanula and Sullivan, 2008). Based on comparisons of volatile chemicals emitted from chipped redbay wood, manuka oil, and phoebe oil, Hanula and Sullivan (2008) hypothesized that two sesquiterpenes, α-copaene and calamenene, were likely the primary host attractants.

We evaluated the response of female *X. glabratus* to volatile chemicals released from commercially available manuka and phoebe oil lures, from freshly-cut logs (bolts) of avocado (cultivars representative of the three horticultural races), and from freshly-cut bolts of lychee (*Litchi chinensis* Sonn., Sapindacaeae), a presumed non-host recently found to contain large quantities of α-copaene (Niogret et al., 2011). Studies included two field tests conducted in natural stands of swampbay with known infestations of *X. glabratus,* and several laboratory bioassays to assess attraction and boring behaviors in newly-emerged female *X. glabratus*. In addition, volatile collections and gas chromatography-mass spectroscopy (GC-MS) were performed to quantify the sesquiterpene content of the substrates used in field and laboratory tests. These studies were designed to address three principal questions: (1) Does *X. glabratus* show a preference among the three races of avocado; (2) Are essential oil lures competitive with host avocado wood for capture of *X. glabratus*; and (3) Does α-copaene content account for the patterns of attraction observed with female *X. glabratus*?

Methods and Materials

Field Tests Field trapping experiments were conducted in north-central Florida (Alachua County) at the Lochloosa Wildlife Conservation Area (St. John's River Water Management District). This area consisted of mesic forest

composed of an overstory of slash pine, *Pinus elliottii* Englem., with a mixed middlestory that included numerous swampbay trees exhibiting advanced stages of laurel wilt. Presence of *X. glabratus* and symptomatic *Persea* trees had been observed at this area since 2007 (A. E. Mayfield, personal communication). GPS coordinates for the four corners of the study site were as follows: N29.47416, W82.14974; N29.47354, W82.15018; N29.47673, W82.15099; and N29.47707, W82.15013. Test I was conducted from 7 October to 2 December 2009 and evaluated attraction of *X. glabratus* to six treatments: a commercial manuka oil lure (release rate 50 mg oil/d; Synergy Semiochemicals, Burnaby, BC, Canada), wood bolts from three avocado cultivars ['Simmonds', West Indian race, *P. americana* var. *americana* Mill.; 'Brooks Late', Guatemalan race, *P. americana* var. *guatemalensis* Williams; and 'Seedless Mexican', Mexican race, *P. americana* var. *drymifolia* (Schlect. and Cham.)], bolts from lychee (cultivar unknown), and an unbaited control. Test II was conducted from 5 November to 29 December 2009 (adjacent to test I) and contained a commercial phoebe oil lure (50 mg oil/d; Synergy Semiochemicals) in addition to the six treatments used in test I.

Wood bolts were obtained from the USDA-ARS germplasm collection at the Subtropical Horticulture Research Station (SHRS), Miami, FL, USA 1 day prior to test deployment. At time of collection, the ends of the bolts were coated with wax to prevent desiccation, and then both ends re-cut when used as bait at the start of each test. Trap design consisted of two bolts (5 cm diam. × 15 cm length) wired together and hung vertically with two white sticky panels (23×28 cm, Sentry wing trap bottoms; Great Lakes IPM, Vestaburg, MI, USA) stapled back-to-back to the bottom of the bolts. Paired sticky panels were secured further with several binder clips around the edges. The essential oil lures were deployed in four-unit Lindgren funnel traps (BioQuip, Rancho Dominguez, CA, USA), with a single lure tied to the side of the trap, suspended midway between the second and third funnel. The collection cups were filled with 300 ml of an aqueous solution of 10% propylene glycol (Low-Tox antifreeze; Prestone, Danbury, CT, USA) to retain and preserve captured insects.

Field tests followed a randomized complete block design, with five replicate blocks arranged in a rectangular grid. Each block consisted of a row of traps hung ~2 m high in non-host trees, with a minimum of 10 m spacing between adjacent traps in a row, and with 50 m spacing between rows. Both tests were 8 wk in duration and checked every 2 wk. At each sampling date, the retention solutions and sticky panels were collected, a thin layer (~1 cm) was sawed from the bottom of each bolt (to "renew" release of wood volatiles), the solutions/sticky panels were replaced, and the trap positions were rotated sequentially within each row to minimize positional effects on beetle capture.

All sample collections were sorted in the laboratory at the Subtropical Horticulture Research Station (SHRS), and species of bark and ambrosia beetles were counted, photographed, and stored in 70% ethanol. Specimens removed from sticky panels were soaked overnight in histological clearing agent (Histo-clear II; National Diagnostics, Altanta, GA, USA) prior to storage in alcohol. Beetle identifications were confirmed at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS-DPI, Gainesville, FL, USA), and voucher specimens were deposited at both SHRS and FDACS-DPI.

Laboratory Bioassays Behavioral bioassays were conducted from March to April 2010 at the University of Florida (Department of Entomology and Nematology, Gainesville, FL, USA) by using the same germplasm accessions used in field tests. Choice tests were performed primarily to assess relative attraction of substrates; no-choice tests were performed to document boring behaviors of host-seeking females presented with individual substrates. Both bioassays were designed to document behavioral responses over time. Experimental insects were female *X. glabratus* (≤3 days post-emergence) reared from field-collected logs of *P. borbonia* maintained in the laboratory under a 0:24 hr (L:D) photoperiod at 25±2°C (Brar et al. unpublished). Test baits were wood bolts (5 cm diam. × 15 cm length) cut 24 hr prior to testing, with the cut surfaces left uncoated. This allowed for partial wound healing and prevented a sticky surface at the cut site that could potentially immobilize beetles.

Two-choice tests were performed in arenas consisting of rectangular plastic bins (122 cm length, 61 cm width, 16 cm height) with tight-fitting lids containing screen inserts. The full length of the bin floor was lined with paper to provide a rough surface upon which the beetles could walk. Single bolts of lychee and avocado ('Simmonds' cultivar) were placed at opposite ends of the bin, and 20 female *X. glabratus* were introduced at the center and immediately covered with an inverted glass funnel (6 cm diam.). After 10 min acclimation, the beetles were released by gently removing the funnel, and the test was initiated. The number of beetles on each bolt was recorded at 1, 2, 4, 8, 24, and 48 hr. At 48 hr, the number of beetles that had bored completely into the bolts and the location of the bore site were also recorded. Two-choice tests were replicated five times (run concurrently) by using separate arenas, baits, and beetles, and the position of the bolts was alternated between replications.

Arenas for three-choice tests consisted of the rectangular bins described above, but only the center of the bin floor was lined with a paper disk (60 cm diam.). Single bolts of

the three avocado cultivars were placed in random order around the edge of the paper, and 25 female *X. glabratus* were introduced at the center and allowed to acclimate prior to testing (as above). The number of beetles that had bored completely into each of the bolts and the location of the bore sites were recorded after 24 hr. The experiment was replicated five times (run concurrently) by using separate arenas, bolts, and beetles.

No-choice tests were performed in cylindrical plastic arenas (4.4 l buckets) with screened lids. Each bucket was lined with a paper disk (15 cm diam.) and set up to contain one bolt and 10 female *X. glabratus*. Bolt treatments consisted of lychee, the three cultivars of avocado, and live oak, *Quercus virginiana* (Fagaceae), as a non-host control (Fraedrich et al., 2008; Mayfield et al., 2008). For this bioassay, a beetle was scored positive for boring (considered fully committed to the boring behavior) at the point when full insertion of the pronotum (approximately half the body length, 1 mm) was observed. The number of beetles boring and their location on the bolt were recorded at 2, 4, 6, 8, 24, and 48 hr. No-choice tests were replicated five times for each of the five bolt treatments, by using separate arenas, new bolts, and new beetles for each replicate.

Chemical Collection and Analysis Samples for chemical analysis were prepared by manually rasping the outer layers from freshly-cut branches (5.0±0.5 cm diam.) of lychee, avocado, and live oak, by using methods reported previously (Niogret et al., 2011). Branches of lychee and avocado were collected from the same germplasm accessions used in field tests. Samples consisted of the outer bark and cambial tissue (the lateral meristem including the vascular cambium and cork cambium layers that carry the secondary metabolites). Volatile chemicals were collected from rasped wood (6-g samples, 3 replicates per tree) and from essential oil lures (phoebe and manuka, aged 1 wk in the field, 3 replicates per lure) by using Super Q traps (Analytical Research Systems, Gainesville, FL, USA) according to published methods (Heath and Manukian, 1992; Heath et al., 1993). Samples were spread in a cylindrical glass chamber (4.5 cm diam. × 25 cm length for rasped wood samples; 10 cm diam. × 38 cm length for essential oil lures), purified air was introduced into the chamber (1 l/min), and headspace volatiles were collected for 15 min. Super Q traps were cleaned by soxhlet extraction with methylene chloride for 24 hr and dried in a fume hood prior to each use. Volatile chemicals were eluted from the Super Q adsorbent with 200 µl of high purity methylene chloride (99.5% pure; ACROS, Morris Plains, NJ, USA). An aliquot of C_{16} standard (5 µg) was added to each sample for quantitative analysis. All chemical sampling was performed within 2 hr of collecting the branch material.

Chemical extracts were analyzed by using a gas-chromatograph (ThermoQuest Trace GC 2000, Austin, TX, USA). The column was fused silica, 25 m long, 0.25 mm i.d., DB-5MS phase (J&W Scientific, Agilent Technologies, Santa Clara, CA, USA), programmed from 50 to 130°C at 15.0°C/min, then from 130 to 220°C at 10.0°C/min, and then held at 220°C for 4 min. The column used in the gas chromatograph interface to the mass spectrometer (Agilent Technologies 5975B) was 25 m long, 0.25 mm i.d., DB-5MS phase (J&W Scientific, Agilent Technologies), programmed at 40°C for 2 min, then from 40 to 130°C at 10.0°C/min, then from 130 to 220°C at 20.0°C/min, and then held at 220°C for 4 min. Chemicals were identified by using the NIST mass spectral program version 2.0 d and the NIST/EPA/NIH mass spectral library (NIST05) when Reverse Matches and Matches were >950 and >900‰, respectively. For each sesquiterpene, the Kovats Retention Index (RI) was compared with the RI calculated from synthetic chemicals when commercially available [RI=1358, 1391, 1442, 1477 for α-cubebene (Bedoukian Research Inc., Danbury CT, USA), α-copaene (Fluka Analytical, Stenheim, Germany), β-caryophyllene (Sigma Chemical Co., St. Louis, MO, USA), and α-humulene (Sigma), respectively] or with previously published data [δ-elemene (Singh et al., 2007), β-elemene (Hosni et al., 2008; Karlsson et al., 2009), alloaromadendrene (Tokushima et al., 2010), cadinene (Singh et al., 2007; Ibrahim et al., 2010), and calamenene (Ibrahim et al., 2010)].

Statistical Analysis Results from the field tests and three-choice bioassays were analyzed by analysis of variance (ANOVA) with Proc GLM (SAS Institute, 2001) followed by least significant difference test (LSD) for mean separation ($P<0.05$). The Box-Cox procedure, which is a power transformation that regresses log-transformed standard deviations ($y+1$) against log-transformed means ($x+1$), was used to determine the type of transformation necessary to stabilize the variance prior to analysis (Box et al., 1978). Paired *t*-tests were used to analyze results from the two-choice bioassays, and regression analysis was used to document patterns in boring behaviors observed in the no-choice bioassays (Systat Software, 2006a). For each sesquiterpene, the captures of *X. glabratus* in field test II were compared to the quantity of chemical per substrate by using Pearson product moment correlation (Systat Software, 2006b). Unless otherwise noted, results are presented as mean ± s.d.; probability was considered significant at a critical level of $\alpha=0.05$.

Results

Field Tests Population levels were low at the study site, and the combined trapping results from both field tests

totaled ~600 ambrosia beetles. *Xyleborus glabratus* was the dominant species, comprising 75.4 and 86.2% of the captures in tests I and II, respectively. Non-target captures included 12 species representing four tribes of ambrosia beetles. Data on the diversity of ambrosia beetles captured with lychee, avocado, and essential oil lures are presented elsewhere (Kendra et al., 2011).

In field test I (Fig. 1a), there were differences in capture of female *X. glabratus* among the six treatments

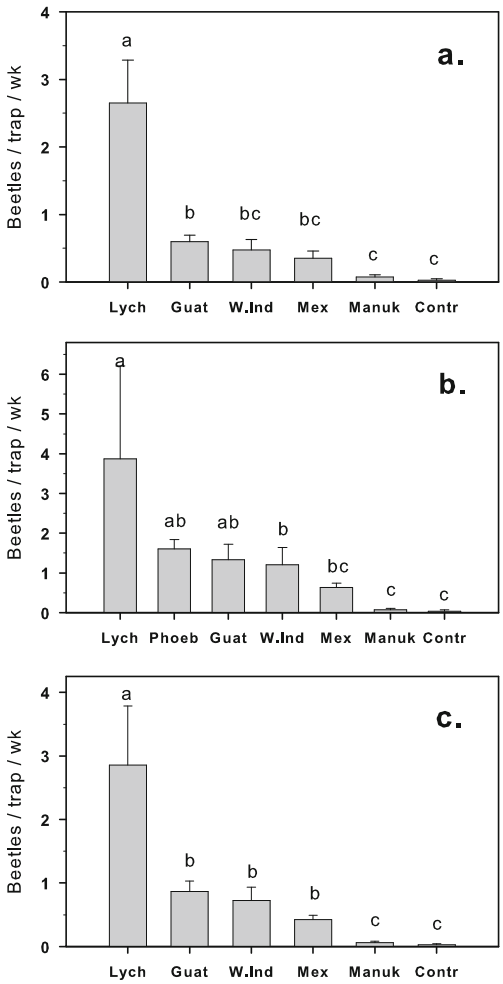


Fig. 1 Mean (±s.e.) captures of female *Xyleborus glabratus* in 8-wk field tests conducted in north-central Florida. (**a**) Test I (N=5) evaluated captures with a manuka oil lure (Manuk); with wood bolts of lychee, *Litchi chinensis* (Lych); and with wood bolts of three cultivars of avocado, *Persea americana*: 'Brooks Late', Guatemalan race (Guat); 'Simmonds', West Indian race (W. Ind); and 'Seedless Mexican', Mexican race (Mex). (**b**) Test II (N=5) evaluated the same treatments as Test I, but also included a phoebe oil lure (Phoeb). (**c**) Combined analysis of the in-common treatments from tests I and II (N=10; There were no interactions between test and treatment for beetle captures, nor an effect caused by test). Commercial lures were deployed in 4-unit Lindgren funnel traps; wood bolts were deployed with sticky traps. Both tests included a control (Contr) treatment that consisted of an unbaited sticky trap. Bars topped with the same letter are not significantly different (LSD mean separation on square root [x+0.5]-transformed data, nontranformed means presented, P<0.05)

(F=16.67; df=5, 29; P<0.001). Traps baited with lychee bolts caught significantly more beetles than any other treatment. Captures with avocado bolts were intermediate, and there were no differences observed among the three cultivars evaluated. Manuka-oil-baited traps captured the fewest beetles, but captures were not significantly different from traps baited with the Mexican or West Indian avocado cultivars. Manuka lure-baited traps captured *X. glabratus* during the first 2 wk only; no beetles were captured with manuka lure-baited traps from wk 3 to 8, despite capture of beetles with all other treatments.

Mean captures of *X. glabratus* were slightly higher during the second field test (Fig. 1b), and there were differences in captures among the seven treatments (F=6.51; df=6, 34; P<0.001). Overall, the hierarchy of captures was similar to that observed with test I. Lychee bolts captured the greatest number of beetles, although not significantly different from captures with the phoebe lure or the Guatemalan avocado. The phoebe lure was also competitive with bolts from all three avocado cultivars. All beetle captures with manuka lure-baited traps occurred within the first 2 wk of the test.

Two-way ANOVA indicated there were no interactions between test and treatment for captures of *X. glabratus* (F=0.58; df=5, P=0.714), nor was there an effect caused by test (F=0.81; df=1, P=0.373). Therefore, data from the two field tests were combined, generating 10 replicate blocks of the six in-common treatments. With the increase in number of observations, there were differences in captures among treatments (F=18.26; df=5, 59; P<0.001) and improved separation of means (Fig. 1c). Captures with lychee bolts were significantly greater than captures with avocado bolts, and captures with avocado were significantly greater than captures with manuka lure, which was comparable to the unbaited control treatment.

Laboratory Bioassays In two-choice tests with bolts of lychee and avocado (Fig. 2), more *X. glabratus* were attracted to lychee at the initial 1-hr and 2-hr observations (t=−2.49, df=8, P=0.038; and t=−2.41, df=8, P=0.043, respectively). By 4 hr, there were equal numbers of beetles on both choices (t=−0.30, df=8, P=0.770). By 8 hr, many of the beetles had left the lychee, but the numbers on avocado remained unchanged. By the end of the 48-hr test, the pattern had reversed and there were more *X. glabratus* on avocado (t=3.236, df=8, P=0.012). With both lychee and avocado, the majority of *X. glabratus* were actively boring into the bolts for all observations taken at or beyond 8 hr. At 48 hr, there were more beetles boring on avocado than on lychee (t=2.467, df=8, P=0.039). A higher percentage of boring occurred on the cut ends of the bolts as compared to the bark surfaces (91.3 and 80.0% for avocado and lychee, respectively).

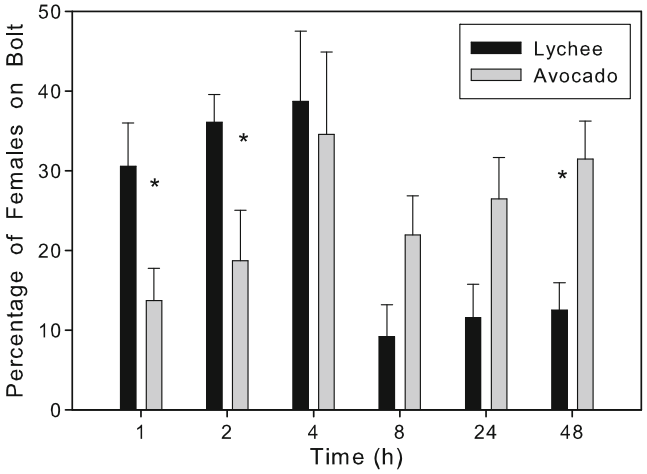


Fig. 2 Mean (±s.e.) percentage of female *Xyleborus glabratus* on wood bolts of lychee, *Litchi chinensis*, or avocado, *Persea americana* cv. 'Simmonds', West Indian race, presented in a 48-hr two-choice bioassay. With both choices, the majority of beetles were boring into the bolts at readings ≥8 hr. Asterisk indicates that mean responses to lychee and avocado are significantly different (*t*-test, $P<0.05$)

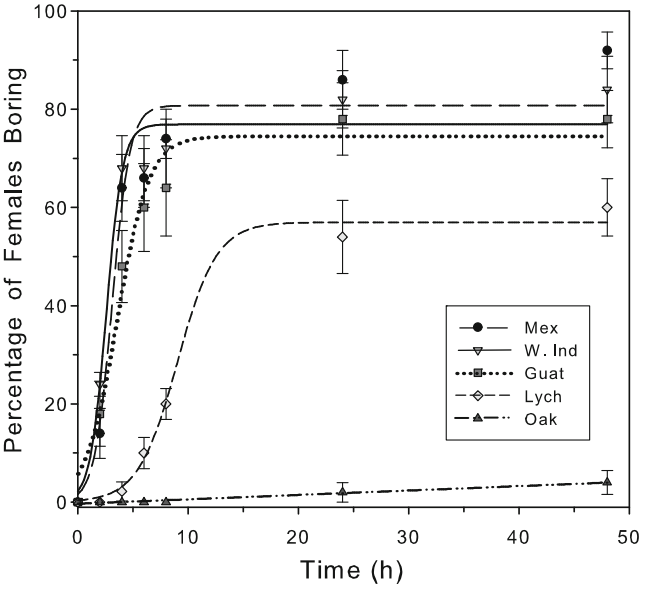


Fig. 3 Mean (±s.e.) percentage of female *Xyleborus glabratus* boring into wood bolts presented in a 48-hr no-choice bioassay. Treatments consisted of three cultivars of avocado, *Persea americana*: 'Brooks Late', Guatemalan race (Guat), 'Simmonds', West Indian race (W. Ind), and 'Seedless Mexican', Mexican race (Mex); lychee, *Litchi chinensis* (Lych); and Live oak, *Quercus virginiana* (L. Oak). Rate of boring was best fit by sigmoidal regression models (see text)

In three-choice tests among the avocado cultivars, the percentage of *X. glabratus* boring into each cultivar after 24 hr was as follows: 31.2 (±16.5)% West Indian; 28.6 (±20.3)% Guatemalan; and 40.2 (±7.0)% Mexican. There was not a significant difference among choices ($F=0.755$; $df=2, 14$; $P=0.491$). The percentage of boring on cut ends of avocado bolts (all cultivars combined) was 64.5%.

The composite results of no-choice bioassays with different bolt treatments are presented in Fig. 3. Regression analysis with sigmoidal models best described the relationships between the time after presentation of avocado or lychee bolts and the percentage of female *X. glabratus* engaged in boring behaviors [Mexican: $y = 80.75/\left(1+e^{-[(x-3.13)/0.80]}\right)$, $R^2=0.951$; West Indian: $y = 76.92/\left(1+e^{-[(x-2.53)/0.76]}\right)$, $R^2=0.971$; Guatemalan: $y = 74.47/\left(1+e^{-[(x-3.56)/1.44]}\right)$, $R^2=0.973$; and lychee: $y = 56.99/\left(1+e^{-[(x-9.04)/1.78]}\right)$, $R^2=0.995$]. Boring was initiated quickly with the avocado cultivars, with the maximum percentage reached by ~8 hr. With lychee bolts, females spent more time walking on the wood substrate before choosing a site and committing to boring. This resulted in a delay (relative to avocado) in boring response with the maximum percentage not reached until ~15 hr. At the conclusion of the test, the mean percentage of boring was as follows: 80.75 (±5.16)% Mexican; 76.92 (±3.42)% West Indian; 74.47 (±3.84)% Guatemalan; and 56.99 (±1.65)% lychee. Few beetles responded to the bolts of live oak (4.0±1.09%), and the responses observed were likely to be thigmotactic rather than olfactory or gustatory-guided behaviors related to host selection. Response of *X. glabratus* to live oak was best fit by linear regression ($y=-0.326+0.09x$, $r^2=0.976$). At 48 hr, there were differences in boring among the five bolt treatments ($F=32.863$, $df=4, 24$; $P<0.001$). There was no difference in percentage of boring among the three avocado cultivars. Percentage of boring in avocado was greater than in lychee, and boring in live oak was significantly less than in all other treatments. The percentage of boring on cut ends (all bolt treatments combined) was 71.9%.

Chemical Analysis A total of 29 volatile chemicals (detected at quantities ≥0.5 μg) were isolated and identified by Super Q collections and GC-MS analysis. Of these, there were nine sesquiterpenes found in common (Table 1). Sesquiterpene content varied both qualitatively and quantitatively among the samples (Fig. 4), but only three chemicals were positively correlated with captures of *X. glabratus* in field test II. These included α-copaene (*Pearson coeff.*=0.427, $P=0.010$; Fig. 4, peak 3), β-caryophyllene (*Pearson coeff.*=0.395, $P=0.019$; Fig. 4, peak 5), and α-humulene (*Pearson coeff.* = 0.514, $P=0.002$; Fig. 4, peak 6). Volatiles from lychee wood (Fig. 4a) contained large amounts of all three chemicals correlated with beetle captures, but also contained a large peak (identification unknown; RI=1504, quantity=9.21±2.10 μg) not detected in other samples. Wood from the West Indian avocado (Fig. 4c) was lower in α-copaene compared to the other two cultivars. Wood from live oak (Fig. 4e) was low in both α-copaene and α-humulene, but had moderate levels of β-caryophyllene. Calamenene was not correlated with beetle

Table 1 Quantity of volatile sesquiterpenes emitted from wood bolts of lychee, *Litchi chinensis*, three cultivars of avocado, *Persea americana*, and from commercial lures of phoebe oil and manuka oil

Chemical	RI[a]	Lychee	Avocado Cultivars			Live Oak	Essential Oil Lures	
			Guatemalan 'Brooks Late'	West Indian 'Simmonds'	Mexican 'Seedless Mex.'		Phoebe[b]	Manuka[b]
δ-Elemene[c]	1347	0.13±0.09	0.87±0.47	0.67±0.14	0.22±0.04	0.26±0.05	0.10±0.03	0.01±0.00
α-Cubebene[d]	1361	0.41±0.03	11.43±1.82	2.26±0.71	1.28±0.25	0.10±0.07	0.55±0.18	3.68±0.22
α-Copaene[d,e]	1394	23.61±3.46	12.93±4.99	2.81±0.60	15.95±4.95	1.06±0.75	8.62±2.76	4.82±0.18
β-Elemene[c]	1403	0.23±0.08	4.09±1.44	1.24±0.33	1.69±0.41	1.27±0.26	0.63±0.17	0.91±0.05
β-Caryophyllene[d]	1443	15.79±4.66	14.39±7.63	1.83±0.44	5.16±0.82	5.18±0.30	0.20±0.08	1.59±0.19
α-Humulene[d]	1477	3.58±0.54	1.30±0.66	0.38±0.09	0.39±0.07	0.18±0.10	0.44±0.11	0.15±0.03
Alloaromadendrene[c]	1482	0.24±0.09	3.55±1.81	0.03±0.01	0.78±0.20	1.61±0.46	1.14±0.11	0.35±0.02
Cadinene[c]	1530	2.28±0.52	1.61±0.74	0.80±0.10	2.98±0.82	0.82±0.34	1.44±0.12	0.50±0.05
Calamenene[c]	1537	0.15±0.01	1.60±0.74	0.04±0.00	0.12±0.10	0.10±0.06	0.33±0.03	5.88±0.38

Table entries are in μg (mean ± s.d.)

Volatiles were isolated from 6 g of rasped wood samples by Super Q collection and then analyzed by GC-MS (DB-5MS column)

[a] Mean Kovats Retention Index calculated from 3 replicate runs per sample

[b] Emissions measured from lures 1 wk old.

[c] Identification based on comparison with RI from published reports (see text)

[d] Identification based on comparison with RI from synthetic chemicals (see text)

[e] Co-eluted with ylangene

captures and was a major component only in the manuka lure sample (Fig. 4g, peak 9).

Discussion

The current detection and monitoring system for *X. glabratus* in the United States is the Lindgren funnel trap baited with manuka oil lures (Lindgren, 1983; CISEH, 2010). Results from our field tests indicated that manuka lures were not competitive with the volatiles released from host avocado wood. In the original studies conducted by Hanula and Sullivan (2008), neat manuka oil dispensed from glass vials was as attractive to *X. glabratus* as wood bolts from redbay. The commercial lure evaluated in our tests released ~50 mg/d of manuka oil (total oil loss determined gravimetrically, per manufacturer specifications). This may not be adequate for extended capture of *X. glabratus*, since no beetles were caught with manuka lures after 2 wk in either test. Two additional field tests conducted in 2010 (Kendra et al. unpublished) confirmed a maximum field life of 2–3 wk for this lure in Florida. Further work is needed to determine the effective loading dosage of manuka oil as well as the release rates of individual attractive components (i.e., α-copaene, calamenene), as has been quantified for other insect lures (Heath et al., 2007). Published chemical analyses of manuka oil have shown a high degree of variability among extracts from trees of different geographic regions in New Zealand (Porter and Wilkins, 1998), which may also contribute to variability in attraction of *X. glabratus.*

Commercial phoebe oil lures were competitive with host avocado wood for capture of *X. glabratus*, and were effective throughout the entire 8-wk test. Unfortunately, the source of phoebe oil, the Brazilian walnut tree, is a limited resource in the Amazon rain forest and may not be available for future use. Another drawback of both manuka and phoebe lures was their non-specificity for *X. glabratus*. Ambrosia beetles are a large, diverse faunal group in tropical and subtropical regions (e.g., >500 recognized *Xyleborus* spp. from the neotropics, Africa, and Asia; Rabaglia, 2002), and numerous non-target species were captured in traps baited with essential oil lures in north Florida (Kendra et al., 2011). Once healthy swampbay trees were attacked by *X. glabratus*, the stressed trees were susceptible to further attack by an array of secondary colonizers (primarily species from within the tribe Xyleborini). These secondary species were attracted to the same host-based volatiles as *X. glabratus*, contributing to high non-target captures in field traps (14, 25, and 85% non-target captures at three field sites in Alachua and Marion Counties, FL). A similar diversity of ambrosia beetles (~15 species) has been observed to attack stressed avocado trees in south Florida (Peña et al. unpublished), and manuka-baited Lindgren traps deployed in avocado groves (Miami-Dade County, FL, USA) captured high numbers of

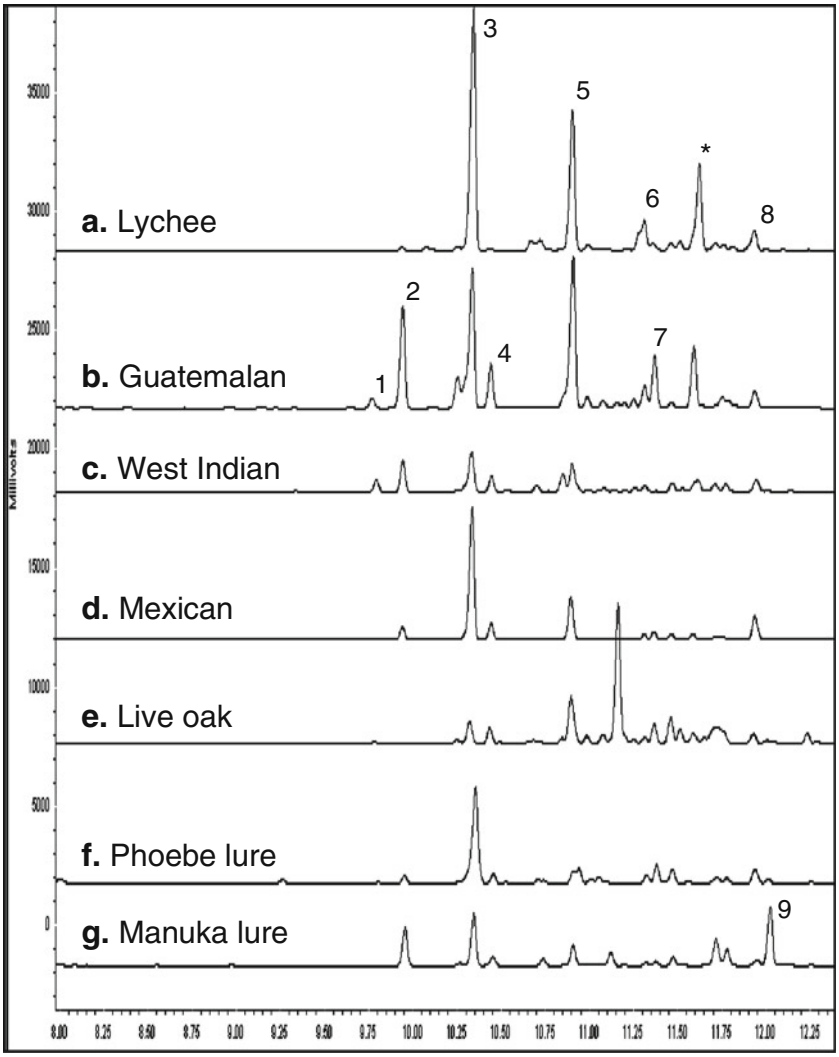


Fig. 4 Representative GC-MS analyses of sesquiterpenes obtained by Super Q collections from wood of (**a**) lychee, *Litchi chinensis*, from wood of three cultivars of avocado, *Persea americana*: (**b**) 'Brooks Late', Guatemalan race, (**c**) 'Simmonds', West Indian race, and (**d**) 'Seedless Mexican', Mexican race; from wood of (**e**) live oak, *Quercus virginiana*, and from commercial lures of (**f**) phoebe oil and (**g**) manuka oil. Peak identifications are as follows: 1) δ-elemene, 2) α-cubebene, 3) α-copaene, 4) β-elemene, 5) β-caryophyllene, 6) α-humulene 7) alloaromadendrene, 8) cadinene, 9) calamenene, and * unidentified chemical (RI=1504)

Ambrosiodmus obliquus (LeConte) and *Hypothenemus* spp. (Kendra et al., 2011).

Two trap types were used in our field tests evaluating captures of *X. glabratus* with attractive substrates: funnel traps with the formulated lures of manuka and phoebe oil; and sticky traps with the wood bolts of avocado and lychee. In an initial field test conducted in Marion County, FL, USA (Kendra et al., 2011), few ambrosia beetles were captured with funnel traps baited with wood bolts. At the recommendation of A. E. Mayfield (USDA-Forest Service, Asheville, NC, USA), we changed our trap design to a sticky panel for evaluation of host wood in the current tests. However, in our experiments, funnel traps were used for deployment of commercial lures rather than sticky panel traps because our goal was to evaluate the efficacy of the current monitoring tool for detection of *X. glabratus.*

Our study provided no evidence that female *X. glabratus* show a preference among the horticultural races of avocado. There were no differences in attraction to bolts from the three cultivars presented in field tests nor in the three-choice bioassays, despite differences in chemical profiles evident from GC analysis. Nor was there a difference in percentage of boring (interpreted as host recognition/acceptance) among the cultivars presented in no-choice tests. Mayfield et al. (2008) evaluated four different cultivars ('Hass', Guatemalan-Mexican hybrid; 'Monroe', Guatemalan-West Indian hybrid; 'Winter Mexican' Mexican; and 'Catalina', Guatemalan-West Indian hybrid) as young potted trees in no-choice tests and found no differences in boring of *X. glabratus* or in transmission of the *Raffaelea* pathogen. As a generalist on woody hosts in the Lauraceae, *X. glabratus* did not appear to distinguish among differences in avocado genotypes (intraspecific variation in secondary metabolites). In all of our laboratory bioassays (and in avocado bolts deployed in field tests), we observed that the majority of boring took place along the cut surface of the bolts, suggesting that host trees would be most susceptible to attack by *X. glabratus*

immediately following pruning activities, when there would likely be high release of host volatiles.

Of the wood substrates evaluated, lychee was the most attractive to dispersing female *X. glabratus,* outcompeting avocado in field tests. In two-choice bioassays, lychee bolts were more attractive than avocado initially, but over time a higher percentage of females bored into avocado bolts. This response suggests that lychee wood provides an initially more powerful olfactory/visual cue than avocado; but after settling on lychee, it may be a suboptimal substrate, and thus the beetles continue to forage until they find a more suitable host (avocado) for long-term settling based on other sensory modalities, including gustation. However, when not given a choice, more than half of the females tested did bore into lychee bolts. Although not in the family Lauraceae (like all confirmed U.S. hosts of *X. glabratus*), lychee should be evaluated for host status and susceptibility to laurel wilt. Since lychee is another subtropical fruit tree grown in the same areas as commercial avocado groves, lychee could serve as a potential reservoir for *Raffaelea* even if it is not susceptible to laurel wilt. The latter would not be unexpected since lychee is native to Southeast Asia and occurs sympatrically with endemic populations of *X. glabratus*. In its native regions, *X. glabratus* is not restricted to the family Lauraceae, and has been recorded from hosts in the Dipterocarpaceae, Fagaceae, and Fabaceae, in addition to Lauraceae (Rabaglia et al., 2006).

Chemical analysis of our test substrates identified three sesquiterpenes that were correlated positively with field captures of *X. glabratus*—α-copaene, β-caryophyllene, and α-humulene. β-Caryophyllene and α-humulene (synonymous with α-caryophyllene) have not been evaluated previously for *X. glabratus*, and need further study as potential attractants. The high levels of α-copaene measured from lychee wood support the conclusion of Hanula and Sullivan (2008) that this sesquiterpene functions as a primary host attractant. The same authors identified calamenene as an attractant in native redbay trees; however, calamenene was detected at low levels in our wood substrates (particularly the West Indian avocado) and did not appear to play a role in attraction to avocado or lychee. Quantification of calamenene was also low in the phoebe oil lure that had been aged in the field for 1 wk. Direct evaluations of α-copaene and calamenene as field lures have yet to be done, due to lack of commercial availability in quantities suitable for field tests. Interestingly, α-copaene, β-caryophyllene, and α-humulene have also been implicated as potential attractant semiochemicals for males of the Mediterranean fruit fly, *Ceratitis capitata* (Wied.) (Diptera: Tephritidae) (Niogret et al., 2011, and references therein). In addition to sesquiterpenes, we detected two monoterpenes, α- and β-pinene, in significant quantities from the avocado cultivars, but not from lychee or live oak (data not shown). Pinenes are known attractants for other species in the Xyleborini, including *Xyleborus pubescens* Zimmermann (Miller and Rabaglia, 2009) and *Xyleborinus saxesenii* (Ratzeburg) (Petrice et al., 2004), and may function as host (Lauraceae) attractants for *X. glabratus* as well.

The unique peak detected in lychee (RI=1504) also needs further evaluation. Unfortunately, the chemical could not be identified even tentatively in this study due to lack of consistent matches in the NIST mass spectral library. Further work is needed for structural confirmation of the chemical and for evaluation of its potential role as an attractant (or possible synergist to α-copaene). Insight into potential attractants for *X. glabratus* may be gained from comparative studies of substrates like lychee and known hosts, including analysis of sesquiterpene content and evaluation of relative attractiveness in field tests or bioassays. However, identification of specific attractants could be facilitated by coupled electroantennography-chromatographic separation (electroantennal detection or EAD) of host volatiles. Future research efforts will be directed toward applying EAD techniques to *X. glabratus* for evaluation of α-copaene, calamenene, β-caryophyllene, α-humulene, and the unidentified peak from lychee.

In summary, our study revealed that the standard detection system for *X. glabratus* (Lindgren funnel trap baited with manuka oil lures) is suboptimal, apparently due to limited longevity of the field lure. Phoebe oil lures were more effective, but their future availability is uncertain. Female *X. glabratus* showed no preference among avocado cultivars (West Indian, Mexican, and Guatemalan races) and preferred to bore into freshly-cut surfaces. This suggests that avocado trees would be most susceptible to attack immediately following pruning activities. Chemical analysis of wood substrates provided supporting evidence that α-copaene is a primary host attractant. Volatiles from lychee wood were more attractive to *X. glabratus* than volatiles from host avocado wood, and lychee may be a source of novel attractants for development of improved detection and control strategies for *X. glabratus*.

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All sawflies sequestering plant secondary metabolites are considered specialists in that they feed on a narrow set of plant species belonging to one genus or family. A restricted diet is also typical for sawflies taken as a whole, with the exception of members of the subfamily Tenthredininae (Tenthredinidae), which includes both specialists and generalists (Taeger et al., 1998; Lacourt, 1999). Little is known about the sequestration ability of Tenthredininae larvae. The iridoid glycoside catalpol was detected in total extracts of larvae, prepupae and exuviae of *Tenthredo grandis* (Tenthredininae), a specialist on *Chelone glabra* (Scrophulariaceae) (Bowers et al., 1993). In the current study, we analyzed several organs from immature stages of *Tenthredo zonula* Klug, a specialist on common St. John's wort *Hypericum perforatum* L. (section *Hypericum*, Hypericaceae). A field population of *T. zonula* was found on common *H. perforatum*, but also on hairy St. John's wort (*Hypericum hirsutum* L., section *Taeniocarpium*). To our knowledge, this represents the first observation that the latter species also serves as a host plant for *T. zonula*.

The contemporary use of *H. perforatum* is as an alternative herbal treatment for mild to moderate depression in North America and Europe. This has resulted in its increased cultivation in several countries (e.g., Canada, the United States, Germany, Poland, France) and collection from the wild in others (e.g., Romania, Serbia, Hungary). The naphthodianthrones hypericin and pseudohypericin, which have been isolated only from certain species of *Hypericum*, along with flavonoid glycosides such as rutin, hyperoside, isoquercitrin, and quercitrin, may contribute to the bioactivity of the extracts (Nahrstedt and Butterweck, 2010). Flavonoid glycosides are found in both leaf and floral tissues of *Hypericum* species, and make up 2-4% of the dry weight (Hölzl and Petersen, 2003). In *Hypericum*, exudate-containing glands appear as lines or dots, and are of two types: translucent (pale yellow to amber in color) and dark (red to nearly black), found variously on the stems, leaves, sepals, petals, and the anther connective (Robson, 2002). Anatomical and histochemical studies of *H. perforatum* have shown that the translucent glands are actually subepidermal cavities, lined with two layers of flattened, thin-walled secretory cells (Ciccarelli et al., 2001a). Evidence suggests that essential oil components and acylphloroglucinol derivatives (i.e., hyperforin) are biosynthesized in these secretory cells and exuded into the cavities (Soelberg et al., 2007). Dark glands (more correctly called "nodules"), meanwhile, are specialized clusters of cells containing wax and the intensely red naphthodianthrone pigments hypericin and pseudohypericin (Ciccarelli et al., 2001b). The concentrations of the naphthodianthrones vary according to the species, but generally range from 0.1-0.3% of the dry tissue weight (Hölzl and Petersen, 2003). *Hypericum perforatum* plants have black nodules scattered on the leaf, petal, and sepal surface, within the leaf, petal and sometimes sepal margin, and on the stamen connective tissue (Robson, 2002). On *H. hirsutum* plants, reddish nodules appear occasionally on the stems; and black nodules are scattered on the leaf and petal surface, as well as within the sepal and petal margin. Those on the sepal margin in this species can be either sessile or on cilia, while in *H. perforatum*, they are always sessile (Robson, 2002, 2010).

The localization of the naphthodianthrones in tissues of *Hypericum* is of interest because these compounds can cause phototoxic effects in grazing insects and mammals that feed on the plants (Knox and Dodge, 1985a). These compounds are photoreactive and when activated by light they catalyze the formation of singlet oxygen, which has damaging effects on enzymes (i.e., inactivation), cell membranes (i.e., disruption through lipid peroxidation) and DNA tertiary structure (Knox and Dodge, 1985b; Berenbaum, 1987). Insects such as leaf beetles and moth caterpillars that feed on *Hypericum* possess behavioral and physiological adaptations that prevent these damaging effects (Larson, 1986; Sandberg and Berenbaum, 1989; Aucoin et al., 1990, 1991, 1995; Duffey and Pasteels, 1993; Guillet et al., 2000).

We observed larvae of *T. zonula* feeding on *H. perforatum* and *H. hirsutum*, actively consuming leaf tissues containing both translucent glands and dark nodules. We were interested in exploring whether this species sequesters the bioactive metabolites from its host-plant, and if so, in which organs and what amounts. Furthermore, we proposed to compare the physiology and ecology of *T. zonula* with that of other insects that feed on *Hypericum*. We determined the amounts of specific flavonoid glycosides (rutin, hyperoside, isoquercitrin, and quercitrin), as well as the naphthodianthrones hypericin and pseudohypericin, in *H. perforatum* and *H. hirsutum*, and analyzed the distribution of these compounds in several organs of larvae and prepupae of *T. zonula*.

Methods and Materials

Plant and Insect Material A population of *T. zonula* was found in the field (Dinant, Belgium; July 2007) feeding on sympatric *H. perforatum* and *H. hirsutum* plants, which grew in monospecific or mixed clusters on the logged edge of a forest. All plant and insect samples used were collected in this location and were identified by using the taxonomic keys published in Robson (2002, 2010) and Lorenz and Kraus (1957), respectively.

Ten aerial branches of *H. perforatum* and six of *H. hirsutum* were collected in the field (on July 05, 2007), and individually marked. The cut branches were placed in

water, while one additional plant of each species was transplanted to a pot. All plants were brought to the laboratory and maintained at 10°C and exposed to natural daylight conditions. For chemical analysis, 5–10 cm long twigs (comprising leaves and flowers) were taken from each of the 10 *H. perforatum* and 6 *H. hirsutum* branches on three dates (July 6th, 13th, and 20th) as well as from the 2 potted plants. Twigs were dried in the dark to a moisture content of less than 2% and stored at room temperature until extraction was conducted. We established that 100 mg of dried plant material were equivalent to 1 g of fresh plant material (1:10 w/w). Voucher specimens of the plants are deposited at the Institute for Pharmaceutical Sciences (Karl-Franzens-University, Graz, Austria).

Tenthredo zonula larvae (Fig. 1a) were collected in the field by gently beating plant clusters above a square net placed horizontally on the ground. Larvae were approximately four times more numerous on *H. perforatum* than on *H. hirstutum*. The density of the larvae on the plants was variable: ca. 50-70% of plant clusters had 1–3 larvae, while the others had none. A few prepupae were collected as well, which indicates that this stage (Fig. 1b) remains without feeding on the plant for a period of time, before pupation in the soil. Most prepupae used in this study, however, were obtained by rearing the field-collected larvae (Fig. 1c). The general feeding behavior of the sawflies was observed while larvae were reared for 2–6 d on leaves taken from plant material from both cut aerial branches and potted plants (see above) until the last larval instar, or prepupal stage. For chemical analyses, 4 to 7 individuals were pooled when they reached either the last or penultimate larval instar, or prepupal stage, whereas excrement from more numerous larvae, at several instars, was combined as one sample. Hemolymph droplets were collected in glass capillaries by gently piercing the integument with forceps, and were deposited in vials after measuring the volumes. Average amounts of hemolymph per individual were 8 µl for larvae and 7 µl for prepupae, but the maximal amount collected from an individual (i.e., 13 µl and 11 µl, respectively) was used to calculate their amounts in µg/individual; values in µmol/g fresh weight (FW) were obtained by considering 1 µl hemolymph as equivalent to 1 mg FW. The individuals were frozen prior to the dissection, at 4°C, of several organs: the digestive tract (its anterior and median parts); the salivary glands; and miscellaneous organs (i.e., integument, muscles, and fat body). The salivary glands (Fig. 1d) were sometimes absent or poorly developed and were discarded. The prepupa is a non-feeding stage, so its digestive tract is empty and was not collected. All samples were immediately stored in the dark, at −20 or −30°C until chemical analysis. Voucher specimens of sawfly immature stages are deposited at the Royal Belgian Institute of Natural Sciences (Brussels, Belgium).

HPLC Analysis Ammonium acetate and glacial acetic acid, as well as acetonitrile (ACN) and methanol (MeOH), were of HPLC grade and purchased from Carl Roth GmbH (Karlsruhe, Germany). Dried plant material was ground to a

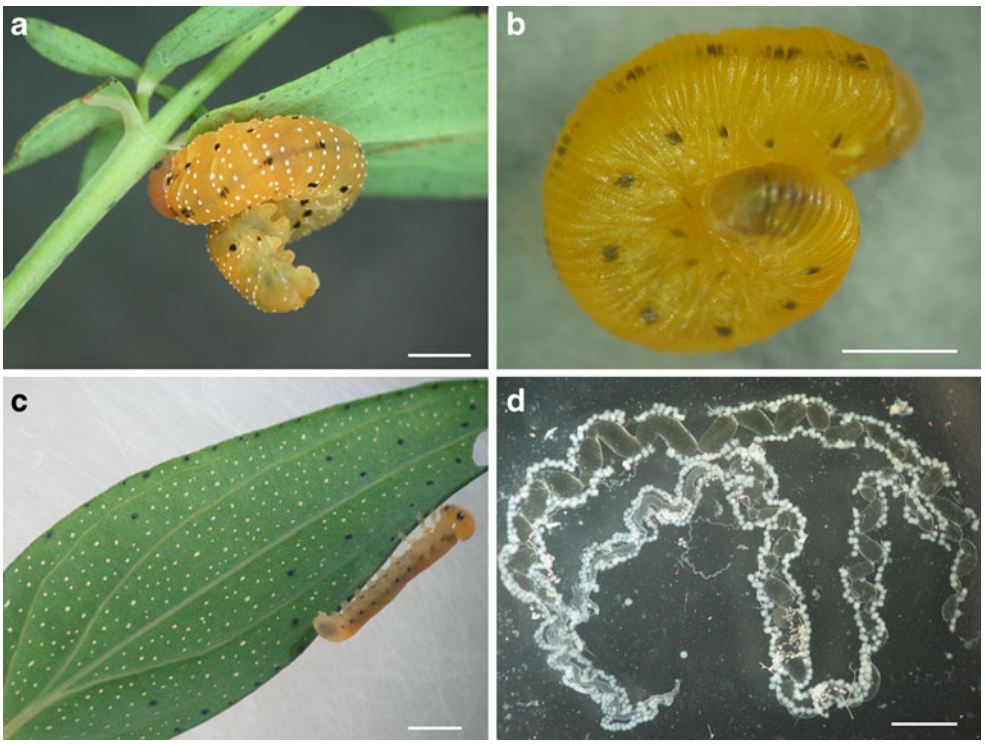


Fig. 1 Sawfly *Tenthredo zonula*. **a** Last instar larva attached to the underside of a leaf of *Hypericum perforatum*; when not feeding, the larva maintains its abdomen typically curled. **b** Prepupal stage; a prepupa never feeds. **c** Young larva on a leaf of *H. perforatum* where translucent and dark glands are visible on its underside. **d** Dissected salivary glands from a prepupa. Bar=2 mm. Photographed by J-L Boevé

fine powder and 250 mg of the powder were extracted four times with 2.5 ml aliquots of MeOH with sonication for 10 min. After 5 min centrifugation at 792 x *g*, the extracts were combined in a 10-ml volumetric flask and filled up to the final volume with MeOH. A 1 ml subsample of this solution was filtered through a 0.45 μm PTFE filter (Carl Roth GmbH), dried under nitrogen, and stored in an amber glass vial at −20°C until analysis, at which time each sample was re-dissolved in 1 ml MeOH. Each insect sample (hemolymph, digestive tubes, salivary glands, and miscellaneous organs) was extracted with 1 ml MeOH with sonication for 10 min and centrifuged for 5 min at 792 x *g*. The clear supernatants were pipetted into 1-ml volumetric flasks and filled to the final volume with MeOH, filtered through a 0.45 μm PTFE filter (Carl Roth GmbH), and directly analyzed. Each sample solution was injected in triplicate.

The identity and purity (>95%) of rutin, hyperoside, isoquercitrin, quercitrin, pseudohypericin, and hypericin standards (Fig. 2) (Chromadex, Inc., Irvine, CA, USA) were confirmed by chromatographic (high pressure liquid chromatography-diode array detection, HPLC-DAD), spectral (1D- and 2D-nuclear magnetic resonance, NMR), and electrospray ionization mass spectrometry (ESIMS) methods. Stock solutions of each standard were prepared at concentrations of 1.0 mg/ml in MeOH and serial dilutions were made with MeOH. Within the range of concentrations injected (0.025-100 μg/ml), the detector response was linear (correlation coefficient R^2=0.998).

All data were recorded and processed using Agilent 1100 Chemstation software (Agilent Technologies, Santa Clara, CA, USA). HPLC analysis was performed on an Agilent 1100 Separations Module equipped with a DAD (Agilent Technologies), using a Synergi MAX-RP 80 Å column (150 x 2.1 mm, 4 μm particle size) from Phenomenex (Torrance, CA, USA) at a flow rate of 300 μl min^{-1}, 5 μl injection per sample (Ganzera et al., 2002). Briefly, the mobile phases consisted of 10 mM ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid (A) and a 9:1 mixture of ACN and MeOH (B). Gradient elution was performed as follows: linear gradient from 13% B to 17% B over the first 10 min, followed by a gradient to 100% B over the next 25 min, followed by re-equilibration for 7 min. All separations were performed at 40°C. Peaks were assigned by spiking selected samples with standard compounds, and comparison of the UV spectra and retention times. HPLC–DAD/ESIMS (positive and negative modes) was performed on a Thermo Finnigan Surveyor liquid chromatography instrument with Thermo Quest Surveyor photodiode array detector, autosampler, and MS pump, and a Thermo Finnigan LCQ-XP mass detector equipped with an ESI source run by Xcaliber software (Thermo Fisher Scientific, Waltham, MA, USA). Mass spectra were detected and recorded in a scan range of *m/z*=100 to 1,000, using a transfer capillary temperature of 350°C, a spray voltage of 5.00 kV and a sheath gas flow of 70 units.

Results

The amounts of flavonoid glycosides (rutin, hyperoside, isoquercitrin, and quercitrin) and naphthodianthrones (pseudohypericin and hypericin) measured in *H. perforatum* and *H. hirsutum* are given in Fig. 3. All compounds were found

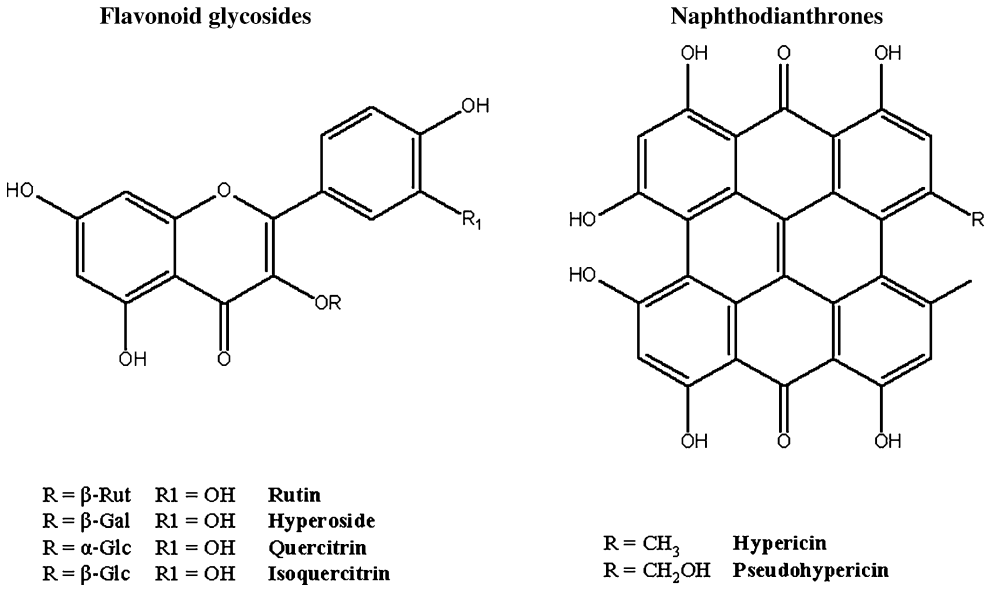


Fig. 2 Flavonoid glycosides and naphthodianthrones found in the plants *Hypericum perforatum* and *H. hirsutum*, and the sawfly *Tenthredo zonula*

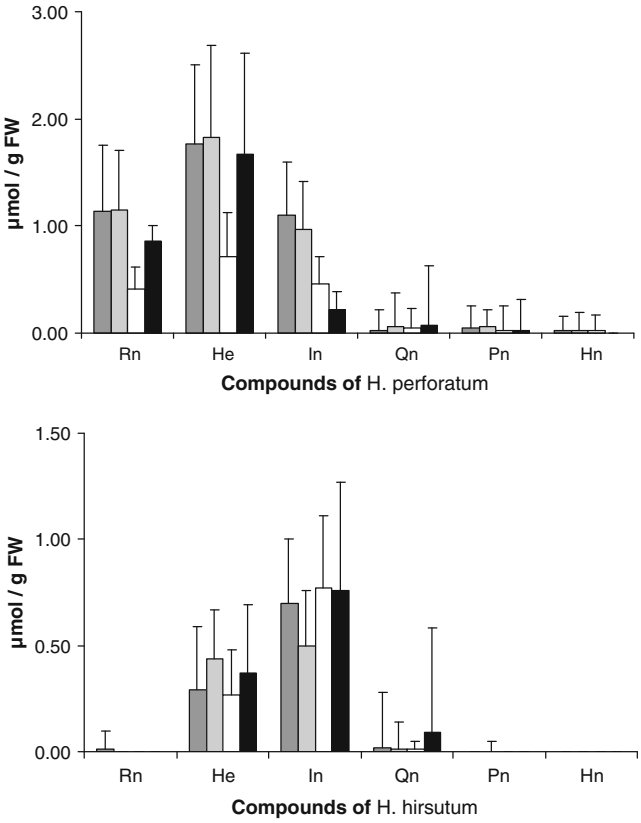


Fig. 3 Concentrations of flavonoid glycosides and naphthodianthrones in *Hypericum* samples. The compounds are: rutin (Rn), hyperoside (He), isoquercitrin (In), quercitrin (Qn), pseudohypericin (Pn), and hypericin (Hn). Values are given in µmol/g FW and as means±SD, $N=3$ technical replicates for each plant sample. From 10 *H. perforatum* and 6 *H. hirsutum* branches having been cut in the field and maintained at the laboratory, leaves and inflorescences were taken from them after 1, 8, and 15 d (i.e., *dark grey*, *light grey*, and *white bars*, respectively, for each compound), and from one potted plant of each species (*black bar*)

in both species, although not in every sample of each species. Two samples of *H. perforatum* differed from the other samples of this species in that they lacked rutin. The major flavonoid glycoside detected was hyperoside in *H. perforatum* (ca. 1.7 µmol/g FW) and isoquercitrin in *H. hirsutum* (ca. 0.7 µmol/g FW). Overlapping peaks were observed in many samples of *H. hirsutum* in the chromatographic region where quercitrin would have appeared, preventing quantification of this compound in some cases. Rutin in *H. hirsutum* and naphthodianthrones in both plant species were infrequently detected, and when detected, they generally were present as minor constituents (0.01 to 0.05 µmol/g FW).

Amounts of the six plant metabolites detected in extracts of specific body parts (hemolymph; digestive tract; salivary glands; pooled miscellaneous organs) of dissected *T. zonula* larvae and prepupae are presented in Table 1. All compounds were detected at some level in the sawflies, although not in all samples or in all body parts. In particular, the concentrations of the flavonoid glycosides were highly variable. The detection of the six compounds was as frequent for larvae reared on one of the cut branches (24 out of 96 potential detections) as for those reared on a potted plant (8 out of 48) ($P=0.29$, Fisher exact probability test), but their detection was associated with the sawfly stage, since a lower frequency of detections was observed in larvae (32 out of 144) than in prepupae (11 out of 18) ($P=0.001$; individuals reared on *H. perforatum*; Table 1).

The highest levels of flavonoid glycosides in the hemolymph were detected in prepupae that had fed during the larval stage on *H. perforatum* (as high as an absolute value of 10 µg/individual=0.45 µmol/g FW of isoquercitrin). In contrast, the four flavonoid glycosides generally were not detected in the hemolymph of larvae although the results for quercitrin were unclear due to overlapping peaks. Flavonoid glycosides also were detected infrequently in the digestive tracts of the larvae. However, the salivary glands of prepupae that had fed as larvae on *H. perforatum* had high concentrations of rutin (41 µg/individual) and isoquercitrin (32 µg/individual). The highest amounts of quercitrin were detected in several of the samples of miscellaneous organs from larvae that had fed upon *H. perforatum*. The naphthodianthrones pseudohypericin and hypericin were detected more often in larvae and prepupae reared on cut branches (15 out of 38 potential detections) than on those reared on potted plants (2 out of 22) ($P=0.01$, Fisher exact probability test). Pseudohypericin was detected most frequently in the digestive tract and/or miscellaneous organs with a maximum for the digestive tract of 0.25 µg/individual. Hypericin was found only in sawflies that had fed upon cut branches of *H. perforatum*.

Pseudohypericin and hypericin were the only screened metabolites detected in the excrement of larvae reared on *H. perforatum*, at concentrations of 0.11 and 0.12 µg/mg DW excrement, respectively, which indicates that the major portions of the ingested naphthodianthrones were excreted without undergoing chemical changes. The presence of both of these naphthodianthrones in the excrement was confirmed by further analysis of this extract using HPLC–DAD/ESIMS (negative mode), but this revealed several additional peaks with UV spectra and mass fragmentation patterns that shared some characteristics with hypericin and pseudohypericin (Fig. 4; Table 2).

Discussion

Amounts of flavonoid glycosides and naphthodianthrones measured in *H. perforatum* and *H. hirsutum* during this study (Fig. 3) are in agreement with those measured during previous studies (Ganzera et al., 2002; Crockett et al., 2005). The primary goal of the current research was to

Table 1 Concentration of flavonoid glycosides and naphthodianthrones in dissected *Tenthredo zonula* immature stages

Sawfly stage/Plant	Qtt	Organ	Rn	He	In	Qn	Pn	Hn
Larva/	39 µl	Hem.	–	–	–	–	–	0.01±0.15
Hypericum perforatum, cut		Hem.[a]						[TR]
	4	Dig. tr.	–	–	–	–	0.02±0.14	–
	3	Sal. gl.	–	–	–	–	–	–
	4	Misc. org.	–	–	–	54.72±13.61	TR	0.01±0.17
Larva/	44 µl	Hem.	–	–	–	–	TR	0.02±0.19
H. perforatum, cut		Hem.[a]					[TR]	[TR]
	5	Dig. tr.	–	–	–	4.10±0.69	–	–
	2	Sal. gl.	2.07±0.45	–	–	–	TR	–
	5	Misc. org.	–	–	–	–	–	–
Larva/	40 µl	Hem.	–	–	–	OV	–	–
H. perforatum, cut		Hem.[a]				[OV]		
	5	Dig. tr.	–	TR	1.84±0.67	–	TR	–
	4	Sal. gl.	–	–	–	–	–	–
	5	Misc. org.	–	–	–	–	TR	–
Larva/	35 µl	Hem.	–	3.25±1.71	–	OV	–	–
H. perforatum, cut		Hem.[a]		[0.20±0.21]		[OV]		
	4	Dig. tr.	–	1.48±0.47	–	–	0.25±0.17	–
	3	Sal. gl.	0.96±0.33	–	10.46±4.23	–	–	–
	4	Misc. org.	–	–	7.96±1.19	62.48±16.72	TR	–
Larva/	46 µl	Hem.	–	–	–	OV	–	–
H. perforatum, potted		Hem.[a]				[OV]		
	6	Dig. tr.	–	2.60±0.69	6.20±1.09	–	0.02±0.19	
	2.5	Sal. gl.	–	–	–	–	–	–
	6	Misc. org.	TR	–	–	–	–	–
Larva/	40 µl	Hem.	–	–	–	–	–	–
H. perforatum, potted		Hem.[a]						
	7	Dig. tr.	–	–	0.86±0.31	–	0.06±0.22	
	3.5	Sal. gl.	–	–	–	–	–	–
	7	Misc. org.	0.86±0.24	–	–	–	–	–
Prepupa/	48 µl	Hem.	4.97±1.17	8.08±1.68	10.03±2.12	–	–	0.04±0.16
H. perforatum, cut		Hem.[a]	[0.15±0.23]	[0.36±0.19]	[0.45±0.31]			[TR]
	6	Sal. gl.	41.19±11.63	–	32.33±12.06	–	–	0.06±0.13
	6	Misc. org.	4.59±1.14	2.38±0.96	–	–	TR	0.01±0.09
Prepupa/	24 µl	Hem.	–	–	–	–	–	–
H. hirsutum, potted		Hem.[a]						
	4	Sal. gl.	–	–	–	–	–	–
	4	Misc. org.	–	–	–	–	–	–
Excrement							0.11±<0.01	0.12±0.01

For eight batches of 4–7 individuals, hemolymph (Hem.) was collected prior to dissection of digestive tract (Dig. tr.), salivary glands (Sal. gl.), or remaining, miscellaneous organs (Misc. org.). The quantity in µl, or number of organs, is indicated in the column headed **Qtt**. Data are given as mean±SD of three technical replicates, in µg/individual. Values of hemolymph are also given in µmol/g FW (Hem.[a], in brackets). If the compound was present at <0.005 of the given concentration unit, it is recorded as *TR*, and if it was not detected it is indicated with a dash (−). *OV* indicates overlapping peaks, which made quantification impossible. The compounds are: rutin (Rn), hyperoside (He), isoquercitrin (In), quercitrin (Qn), pseudohypericin (Pn), and hypericin (Hn)

determine whether these secondary metabolites could be detected in larvae and prepupae of *T. zonula*, and to examine the body distribution of these compounds. While all compounds were observed at some amount in one or more insect samples, our results indicate a patchy distribution in the sawfly body parts. Their occurrence, nonetheless, seems to be influenced by the host-plant's chemical profile and by the developmental stage at which the sawfly specimens were analyzed.

Our study reveals that *T. zonula* is able to sequester flavonoid glycosides, since these compounds were detected in body parts other than the digestive tract. For example,

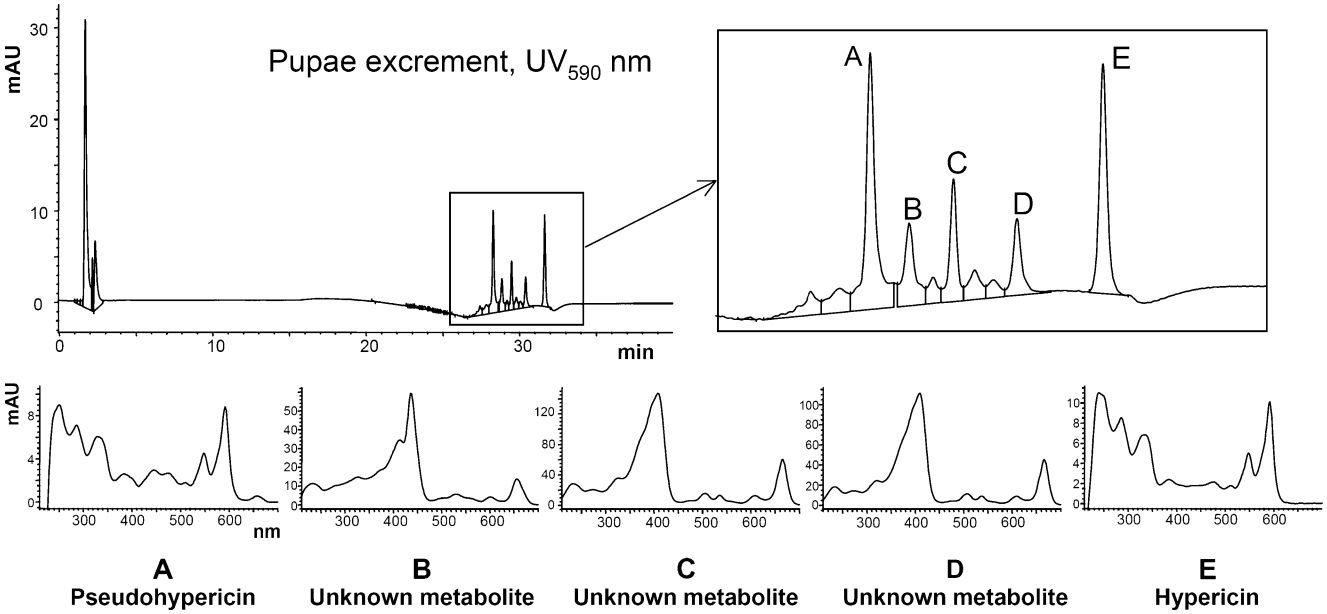


Fig. 4 UV–Vis absorbance spectra from peaks corresponding to the known naphthodianthrones, pseudohypericin **A** and hypericin **E**, and to as-yet-unidentified, but related, lipophilic compounds **B**–**D**

the compounds were found in the larval integument, which formed a main part of the dissected 'miscellaneous organs', as well as in the prepupal hemolymph and salivary glands. Among the isolated organs, only the hemolymph could be weighed, but these measurements allowed comparisons to be made between compound concentrations in plant and sawfly samples. For instance, the concentration of isoquercitrin was about 1 μmol/g FW in *H. perforatum* (Fig. 3) and 0.5 μmol/g FW in the prepupal hemolymph (Table 1). The latter concentration is equal to about 10 μg/individual, and the compound occurred at 32 μg/individual in the salivary glands. As compared to concentrations measured in the plant tissue, isoquercitrin is approximately half as concentrated in the hemolymph of prepupae, and 1.5 times more concentrated in the salivary glands. In the case of prepupae, we conclude that sequestration occurs because this non-feeding stage, which always has an empty digestive tract, can only accumulate plant compounds from the earlier (larval) instars.

Table 2 Negative and positive quasi-molecular and collision induced fragment ions of peaks A-E detected in excrement of *Tenthredo zonula* larvae

Peak	Identity	*m/z* (anions)	Ion	*m/z* (cations)	Ion
A	Pseudohypericin	519	$[M-H]^-$	–	
		487	$[M-H-CH_3OH]^-$	–	
		429	$[M-H-CO_2-CO-H_2O]^-$	–	
B	Unknown	–		594	$[M+H]^+$
		–		534	$[M+H-CH_2=C=O-H_2O]^+$
C	Unknown	–		536	$[M+H]^+$
		–		508	$[M+H-CO]^+$
		–		435	$[M+H-3CO-H_2O]^+$
D	Unknown	–		533	$[M+H]^+$
		–		489	$[M+H-CO_2]^+$
		–		473	$[M+H-CH_2=C=O-H_2O]^+$
E	Hypericin	503	$[M-H]^-$	–	
		487	$[M-H-CH_4]^-$	–	
		433	$[M-H-CH_2=C=O-CO]^-$	–	

Not detected (−)

Tenthredo grandis is the only other *Tenthredo* species that has been studied previously for the possible sequestration of plant metabolites (Bowers et al., 1993). In that study, total extracts of the prepupae contained the iridoid glycoside catalpol at a level up to 3.86% DW, and this compound reached 1.87% DW in leaves of the host plant, *C. glabra*. Thus, both *Tenthredo* species are able to sequester glycosides, at least at the prepupal stage, and we suggest for *T. zonula* larvae that quercitrin is selectively sequestered in the integument, and rutin and isoquercitrin, in the salivary glands. Larval hemolymph may be another storage site for quercitrin, but a methodological problem of overlapping peaks did not always allow quantification. Furthermore, not every flavonoid glycoside was detected in the analyzed batches of larvae. Thus, one should be cautious when discussing the ecological role of these sequestered metabolites as chemical defenses.

Several fates are known for plant metabolites ingested by sawfly larvae, even within one species. For instance, larvae of *Rhadinoceraea nodicornis* (Tenthredinidae, Blennocampinae) sequester some steroidal alkaloids from the host plant, *Veratrum*, in the hemolymph, either directly or after metabolism. However, the larvae excrete or degrade other steroidal alkaloids (Schaffner et al., 1994). In this sawfly species, zygadenine and 3-acetyl-zygadenine are present in the hemolymph at a total concentration of 1.18 μg/mg DW (= 24 μg/individual), whereas 1.77 μg/mg DW of their common precursor, 3-angeloyl-zygadenine, is found in the plant leaves. Larvae of other Blennocampinae species belonging to the genus *Monophadnus* sequester two furostanol saponins in the hemolymph (1.6 and 17.5 μmol/g FW). These compounds are present in the host plant, *Helleborus*, at much lower concentrations (0.008 and 0.268 μmol/g FW) (Prieto et al., 2007). Similarly, glucosinolates and iridoid glucosides are sequestered in the hemolymph by members of the Tenthredinidae genus *Athalia* (Müller et al., 2001; Opitz et al., 2010). Thus, sequestration of plant metabolites occurs repeatedly in Tenthredinidae sawfly larvae. Sequestration renders the hemolymph highly repellent and sometimes toxic to natural enemies such as ants, and provides these species with an efficient chemical defense.

Many classes of secondary metabolites, including naphthodianthrones, can be induced in *H. perforatum* in response to feeding damage from both generalist and specialist herbivores (Sirvent et al., 2003), but also to mechanical wounding such as cutting the plant stem (Lawton and Lamb, 1987). Both naphthodianthrones were found in *T. zonula* larvae that had been fed on cut branches as well as potted plant material, but pseudohypericin was detected more often and in higher amounts than hypericin, and only pseudohypericin was detected in the digestive tract (0.02 to 0.25 μg/individual). The excrement collected from larvae contained 0.11 μg/mg of pseudohypericin, and 0.12 μg/mg of hypericin, but no flavonoid glycosides. These results support the hypothesis that naphthodianthrones are specifically excreted from *T. zonula* larvae. There may be several reasons why only pseudohypericin is detected in the digestive tract. First, the ingested plant material generally contains more pseudohypericin than hypericin. If one assumes a daily consumption of a 36 mm^2 piece of *H. perforatum* leaf tissue (= 18 mg fresh plant material; SLC, personal observation), a larva would ingest about 0.1 – 0.8 μg of hypericin and 0.1 – 1.1 μg of pseudohypericin, which are amounts roughly in the range of those detected in the digestive tract. Second, hypericin may be metabolized to pseudohypericin through the activity of microsomal detoxification enzymes often associated with gut tissues such as cytochrome P450 enzymes, which hydroxylate the substrate (Feyereisen, 1999). Since equivalent amounts of pseudohypericin and hypericin could be identified in the larval excrement, it is likely that hypericin is only partially converted to pseudohypericin in the gut. Third, due to the more apolar nature of pseudohypericin, it does not pass through the gut wall as easily as hypericin (Menn, 1978). However, hypericin as well as pseudohypericin were rarely found in other body parts than the digestive tract, but were clearly present in the excrement.

In the excrement, three additional peaks with similar, but not identical, spectral and mass fragmentation pattern characteristics were seen in the HPLC profiles and were absent from all other insect and plant samples (Fig. 4; Table 2). While the naphthodianthrones hypericin and pseudohypericin exhibit UV maxima at 545 and 590 nm, these compounds displayed intense absorbance peaks between 400–430 nm, and secondary maxima at 660–670 nm. For hypericin, typically observed collision-induced fragment ions of the parent species $[M\text{-}H]^-$ include $[M\text{-}H\text{-}CH_4]^-$ (−16 from parent ion, *m/z* 487) and $[M\text{-}H\text{-}CH_2{=}C{=}O\text{-}H_2O]^-$ (−60 from parent ion, *m/z* 443); for pseudohypericin, such ions include $[M\text{-}H\text{-}CO_2]^-$ (−44 from parent ion, *m/z* 475), $[M\text{-}H\text{-}CH_2{=}C{=}O\text{-}H_2O]^-$ (−60 from parent ion, *m/z* 459) and $[M\text{-}H\text{-}3CO\text{-}H_2O]^-$ (−101 from parent ion, *m/z* 417). The fragmentation patterns observed for peaks B, C, and D showed interesting similarities that could correlate to a loss of $[-CH_2{=}C{=}O\text{-}H_2O]^+$ for peak B (−60 from the parent ion, *m/z* 534); a loss of $[-3CO\text{-}H_2O]^+$ for peak C (−101 from the parent ion, *m/z* 435); and a loss of both $[-CO_2]^+$ (−44 from the parent ion, *m/z* 489) and $[-CH_2{=}C{=}O\text{-}H_2O]^+$ (−60 from the parent ion, *m/z* 473) (Table 2). Although further isolation and structural elucidation are needed to fully clarify the structures of these compounds, it is intriguing to note that several fragment ions seen in the molecular ion pool are 2 mass units greater than several seen in the ion pools of hypericin and pseudohypericin, suggesting that a reduction has taken place. Additionally, the mass of peak D (533)

could be reached by the addition of two methyl groups to hypericin, and the mass of peak C (536), by the addition of a single methyl group to pseudohypericin. These results could indicate either that partial metabolism of hypericin and/or pseudohypericin has occurred in the gut, in which case we hypothesize that the compounds represented at least by peaks C and D present in the excrement extract are metabolites of these compounds, or that the compounds may be the result of post-excretory degradation. These data taken together suggest that *T. zonula* larvae have developed physiological mechanisms that enable them to reduce the absorption of the phototoxic naphthodianthrones by an efficient elimination as a waste by-product.

Other insects, in particular the leaf beetles (e.g., *Chrysolina brunsvicensis, C. geminata, C. hyperici,* and *C. varians*; Coleoptera, Chrysomelidae), possess similar biochemical and physiological adaptations, and also display a specialized feeding behavior that allow them to ingest plant material containing naphthodianthrones without negative effects (Larson, 1986; Duffey and Pasteels, 1993). Analyses of the body distribution of hypericin in *C. brunsvicensis* support the hypothesis that this species contains the majority of hypericin within the gut lumen, and then rapidly eliminates it through the feces (Duffey and Pasteels, 1993). Particular cuticular pigments and, to a lesser extent, cuticle thickness also have been demonstrated to confer protection against the phototoxicity of hypericin in adult *Chysolina* beetles (Berenbaum, 1987; Fields et al., 1990). To block the phototoxic activity of ingested naphthodianthrones, *C. hyperici* is believed to sequester the natural antioxidant molecule β-carotene, in part responsible for the yellow coloration of *Hypericum* petals (Aucoin et al., 1990). Furthermore, elevated levels of antioxidant enzymes such as catalase and glutathione peroxidase have been measured in larvae of the *H. perforatum* specialist *Anaitis plagiata* (Lepidoptera, Lepidopteridae) when fed leaf discs treated with hypericin or reared strictly on *H. perforatum* tissue (Aucoin et al., 1991, 1995). Higher constitutive levels of enzymes such as glutathione reductase and glutathione S-transferase have not been demonstrated for the chrysomelid specialists although they have been measured in two generalist orthopterans, *Tettigonia viridissima* and *Conocephalus discolor*, which only occasionally feed on *H. perforatum* (Guillet et al., 2000). Some insects, such as the larvae of *Platynota flavedana* (Tortricidae), are protected from phototoxic effects through a behavioral adaptation, tying together leaves to form a shaded shelter within which they feed (Sandberg and Berenbaum, 1989). We do not know whether larvae of *T. zonula* have a particularly thickened cuticle or specific cuticular pigments that would potentially protect them against the phototoxic effects of the naphthodianthrones. The larvae do avoid light, displaying a negative phototropism in response to daylight/windows and the cold light source of a binocular microscope (JLB, personal observation). Moreover, in the field, they often are found settling on the underside of a leaf (Fig. 1a), but this behavior is frequently observed in sawfly larvae, regardless of their host plant species. More studies are necessary to fully understand the etho-physiological adaptations in *T. zonula*, and to assess the ecological role of flavonoid glycosides and naphthodianthrones as chemical defense metabolites in this species.

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to the two enantiomers is known (Lanier et al., 1980; Slessor et al., 1985).

Whitten et al. (1988) found that ipsdienol of unknown stereochemistry is a major component of the floral scents of several species of neotropical orchids and one species of aroid (see also Schwerdtfeger et al., 2002). The majority of these species are, or are presumed to be, pollinated by fragrance-collecting male orchid bees (Whitten et al., 1988). Ipsdienol of unknown stereochemistry also has been found in the hind tibial pouches of male euglossines [e.g., *E. tridentata*, (Eltz et al., 1999); *Eulaema bombiformis,* (Zimmermann et al., 2006), *Euglossa cyanura*, T. Eltz, pers. obs.]. These species, among others, have been observed to collect racemic (synthetic) ipsdienol from baits (Williams and Whitten, 1983; Whitten et al., 1988; Ramírez et al., 2002). Among the bee species visiting the synthetic baits, the frequent capture of *Euglossa cyanura* (Whitten et al., 1988) is remarkable, because this species is rarely attracted during standard baiting assays (Roubik and Hanson, 2004).

There is a considerable number of publications on substances attractive to Euglossini and how efficient these are for baiting in particular regions of the neotropics (Ramirez et al., 2002; Roubik and Hanson, 2004). However, it rarely has been investigated whether or not the bees are able to distinguish between different optical isomers, even though several of the known attractants are chiral (see Williams and Whitten, 1983 and discussion). Chirality may add another level to the chemical specificity evident in odors of euglossophilous plants, as well as in the perfumes of male euglossine bees themselves (Zimmermann et al., 2009).

Using gas chromatography coupled with electroantennography (EAG), Eltz et al. (2006) studied the responses of males of three different *Euglossa* species to hindtibial extracts of *E. tridentata* which, among other components, contained ipsdienol of unknown stereochemistry. The antennae of all tested species responded to ipsdienol, suggesting that the bees are able to smell this compound. In the present study, our aim was to find out whether euglossine males can distinguish between (+)- and (−)-ipsdienol, by testing (1) their behavioral preferences, and (2) their peripheral olfactory receptor responses to the enantiomers and racemate. With regard to behavioral preferences, we further sought to elucidate whether the less preferred enantiomer acts as a repellent or as an attractant in the racemate.

Methods and Materials

Chemicals Ipsdienol of three different optical purities was purchased in slow-release devices (bubble caps) from Phero Tech Inc. (7572 Progress Way, Delta, British Columbia, V4G 1E9 Canada): racemic ipsdienol [50:50 mixture of (+)- and (−)-isomers], (+)-ipsdienol (97:3), and (−)-ipsdienol (3:97). Each bubble cap contained 40 mg of the respective isomer(s) and released about 0.2 mg of ipsdienol/day at 25°C (J. P. L. Lafontaine, pers. comm.). In most field bioassays, these bubble caps were used directly as odor releasers (see below). In addition, we used aliquots of racemic ipsdienol (Bedoukian Research Inc.) dissolved in hexane (1 g/ml), and pure hexane (p.a.) as solvent control. Chemicals were kept on ice or in a freezer for most of the time.

Field Tests All field bioassays were conducted during May and June 2009 in forests in the state of Veracruz, Southern Mexico, mainly near the "Estacion Biologica Los Tuxtlas" in the biosphere reserve of Los Tuxtlas (18°30′ N, 95°8′ W), and near the village Poza Azul (17°23′N, 94°11′W). Baits were suspended from vegetation at heights between 1.7 and 2.0 m above ground during standardized time periods (9:30–12:30 and 16:00–17:30). Any bee landing on a bait was captured, and only released again if marked by a dot of acrylic paint on the thorax. This was done to avoid pseudoreplication and to minimize potential local enhancement effects reported in foraging bees and wasps (Jarau and Hrncir, 2009). Bees were caught either by entomological nets or by aspirators.

Baits A bait consisted of three parts: 1) an odor releaser; 2) a nylon net (mesh size ~1 mm) covering the odor releaser to minimize direct contact by bees; and 3) a nylon thread (~20 cm) for suspending the bait. For odor releasers, we either used the bubble caps directly or four sheets of unused coffee filter paper (size: ~9×5 cm) to which we added test solution (see below). If not mentioned otherwise, the nylon net covering the odor releaser was replaced after each experiment. During multiple-bait bioassays, the exact position of baits was swapped every 5 min to minimize position bias.

Single-substance Bioassays One bubble cap of (+)-ipsdienol, (−)-ipsdienol, or racemate was presented singly for periods of 40 min. (N>6 per substance). Additionally, hexane (30 µl on filter paper) was used as a control (N=6).

Multiple-choice Bioassays – Equal Concentrations One bubble cap of each of (+)-ipsdienol, (−)-ipsdienol, and racemate were presented simultaneously for intervals of 40 min. (N=14). The baits were normally placed between 2–5 m from each other (min=50 cm; max=7 m). To test whether or not males would switch to forage for the less attractive ipsdienol enantiomer after the attractive enantiomer was depleted, we occasionally (N=9) continued the bioassay for another 40 min with all but the (+)-ipsdienol bait removed.

Multiple-choice Bioassay – Different Concentration Baits included the same as described above (bubble caps) plus an additional filter paper bait with 30 μl aliquots of racemic ipsdienol in hexane, and a filter paper bait with 30 μl of hexane as a control. Although this was not quantified, the filter paper bait was expected to release much more [order (s) of magnitude more] racemic ipsdienol per unit time than the bubble cap.

Sequential Bioassays We tested whether euglossine males leave chemical cues on visited odor sources that could potentially influence foraging behavior of themselves or conspecifics. We used two baits of the filter paper type, which we presented simultaneously during sequential experiments. Each sequential experiment consisted of two periods: During the first 20 min. we presented one bait containing 30 μl hexane, and another bait with 30 μl racemic ipsdienol. Different from other bioassays and the second phase of the sequential bioassays, we did not capture bees landing on the ipsdienol bait, but allowed males to collect on the mesh until the end of the first period. This allowed for the accumulation of any chemicals left by males on the mesh. During the second period, we again presented two baits for 20 min. This time, both baits contained 30 μl of racemic ipsdienol. Whereas the odor of both baits was new, we reused mesh and clips of the baits from the first period. Consequently, both baits differed in that one had traces of chemical substances left previously by odor-collecting males, while the other (previously unvisited) lacked any such substances.

Electroantennography Antennae of male *E. cyanura and E. mixta* (N=10 individuals each) were cut at the base of the flagellum, and mounted between two glass capillaries containing insect physiological solution and silver electrodes. A Syntech (Hilversum, The Netherlands) signal amplifier (IDAC-232) and Syntech EAG)software were used to amplify and record antennal responses to chemicals. A constant stream of humidified clean air, into which we introduced the test stimuli, flowed over the antennal preparation. Five microliters of test solution (see below) were applied to a strip of filter paper (5×10 mm), and the filter paper placed inside a clean pipette tip. Two hundred microliters of air were puffed through the tip and into the air stream using an electronic pipette (Biohit *e*line 50–1,000 ml). Test substances were applied once per antenna in the same order, except for the hexane, which was applied at the beginning and end of each antennal test series.

Test Solutions (+)-Ipsdienol and (−)-ipsdienol were extracted from the bubble caps with hexane, yielding solutions of 2.31 mg/ml of (+)-ipsdienol, and 1.75 mg/ml of (−)-ipsdienol), as quantified by gas chromatography with flame ionization detection. These were further diluted to 1:10, 1:100, 1:1.000, and 1:10.000. Accordingly, the (+)-ipsdienol stimulus was always at a slightly higher concentration than the respective (−)-ipsdienol stimulus, at all dilutions. For comparison and standardization of response data (see below), we also tested solutions of methyl 2-phenylacetate, eucalyptol (1,3,3-trimethyl-2-oxabicyclo [2.2.2.] octane), eugenol (4-allyl-2-methoxyphenol), *R*-limonene [(*R*)-1-methyl-4-(1-methylethenyl)-cyclohexene], and geraniol [(*E*)-3,7-dimethyl-2,6-octadien-1-ol]. All standard solutions were 0.0667 mg/ml. Standardization in relation to the control stimulus was necessary because *E. cyanura* and *E. mixta* differ slightly in size. As EAG response amplitude is influenced by the size of the antenna (Roelofs, 1984), absolute responses could not be compared between species. Instead, we compared relative responses, standardized in relation to the control stimulus (hexane).

Data Analysis For data from single-substance bioassays, we used the Kruskal–Wallis H-tests, followed by Dunn's test for multiple comparisons. For multiple-choice bioassays, we used one-way ANOVA, followed by a Student-Newman-Keul's multiple comparison test for normally distributed data of equal variance or, in case the assumption of normality was not met, Kruskal–Wallis H-tests (again followed by Dunn's multiple comparison test) or Mann–Whitney rank sum test. For sequential bioassays, we used the Wilcoxon signed-rank tests to test for differences between the periods. The Mann–Whitney rank sum test was applied to test for significant differences within a period. We used the two-side paired t-test for comparing EAG responses (amplitude of rapid negative baseline deflection in mV) to different ipsdienol isomers within species, and the two-side unpaired t-test for comparisons of responses between species. For between-species comparisons we standardized the absolute EAG responses in relation to responses to the set of reference substances (see above, in %), to compensate for differences in size between *E. mixta* and *E. cyanura*. The relative response (%) to an ipsdienol isomer was calculated as the absolute response divided by the mean response to the set of reference substances used. For relative responses to the reference substances themselves, the relative response for each reference substance was computed in the same way, except for a reduced set of remaining reference substances (not including the ipsdienol solutions).

Results

Among the 20+ species of Euglossini occuring in the study area, only two were attracted regularly to our ipsdienol baits, and both had a near-exclusive preference for (−)-

ipsdienol and the racemate (see below). *Euglossa cyanura* (Fig. 1) males were attracted in large numbers at all sites, and consequently were subject to all of the analyses below. Additionally, male *Euglossa tridentata* were regularly attracted to racemic and (−)-ipsdienol at Poza Azul, where they were subjected to multiple-choice and sequential bioassays (in addition to *E. cyanura*). *Euglossa mixta* was never attracted to any ipsdienol bait, irrespective of the time of the day, enantiomeric composition, or baiting locality.

Single Substance Bioassays Male *E. cyanura* were most frequently observed on (−)-ipsdienol (6.83±2.64 SD bees in 40 min) and racemic ipsdienol baits (5.67±1.63 SD bees in 40 min). Only a single male was ever observed to visit an (+)-ipsdienol bait, and no bee came to the control (Fig. 2). Overall, there was a difference among visits to the four stimuli (H=27.9, $d.f.$=3, P<0.001).

Multiple-choice Bioassay – Equal Concentrations Male *E. cyanura* preferred (−)-ipsdienol over racemic and (+)-ipsdienol, when all stimuli were tested simulatenously at the same concentration (H=35.10, d.f.=2, P<0.001; Fig. 3a). When (−)- and racemic ipsdienol baits were removed after 40 min, only a few males behaved opportunistically and switched to the remaining (+)-ipsdienol baits (median=3.85%, 1st quartile=0.00%; 3rd quartile=12.95%, N=9, N=88 bees).

Multiple-choice Bioassay – Different Concentrations When racemic ipsdienol was presented in a high concentration simultaneously with a lower concentration of (−)-ipsdienol from bubblecaps, males of both *E. cyanura* and *E. tridentata* preferred racemic ipsdienol over (−)-ipsdienol (Fig. 3b, c), and neglected the remaining baits. Overall, the number of visiting bees per bait was different (ANOVA $F_{cyanura}$=337.4, $d.f.$=4, P<0.001; ANOVA $F_{tridentata}$= 838.2, $d.f.$=4, P<0.001).

Sequential Bioassays As expected from previous multiple-choice bioassays, there was a clear preference for the racemic ipsdienol bait (bait A in Fig. 4), compared to the control bait to which no bee was attracted (bait B in Fig. 4), for both species (Mann-Whitney $U_{cyanura}$=0, P<001; Mann-Whitney $U_{tridentata}$=0, P<0.001). When ipsdienol was added at the beginning of the second experimental period to the former control bait (bait B in Fig. 4), bees immediately started to land on this bait. Both baits possessed equal amounts of ipsdienol (racemate) and, on average, were visited in similar percentages by both *E. tridentata* and *E. cyanura* males. Thus, any chemical traces of odor-foraging males left on one of the baits had no effect on the subsequent preferences of bees (Mann-Whitney $U_{cyanura}$=24, P>0.10; Mann-Whitney $U_{tridentata}$=19, P> 0.10). Consequently, the percentages of bees that landed on one of the two baits (Fig. 4) changed between the two periods for both species and baits (Wilcoxon Signed Rank Test, P<0.02 for both baits and species).

Electroantennography The EAG results reflected the behavioral results in the sense that *E. cyanura* showed (i) stronger absolute EAG responses to (−)-ipsdienol than to (+)-ipsdienol at all except the lowest concentrations (Fig. 5), and (ii) stronger relative EAG responses to (−)-ipsdienol than *E. mixta* males, again at all except the lowest concentration (Fig. 6b). In contrast, relative EAG responses to (+)-ipsdienol, were not different between the two species at any concentration (Fig. 6a). There was also no difference in relative EAG responses to any of the reference substances between the two *Euglossa* species (unpaired t-test or Mann-Whitney U; P>0.05).

Discussion

We demonstrated that male *Euglossa cyanura* discriminate between optical isomers of ipsdienol by showing that they exhibit a near-exclusive preference for (−)-ipsdienol over (+)-ipsdienol. Corresponding results were obtained for *E. tridentata*, the only other euglossine species attracted regularly to ipsdienol in the study area. This suggests that

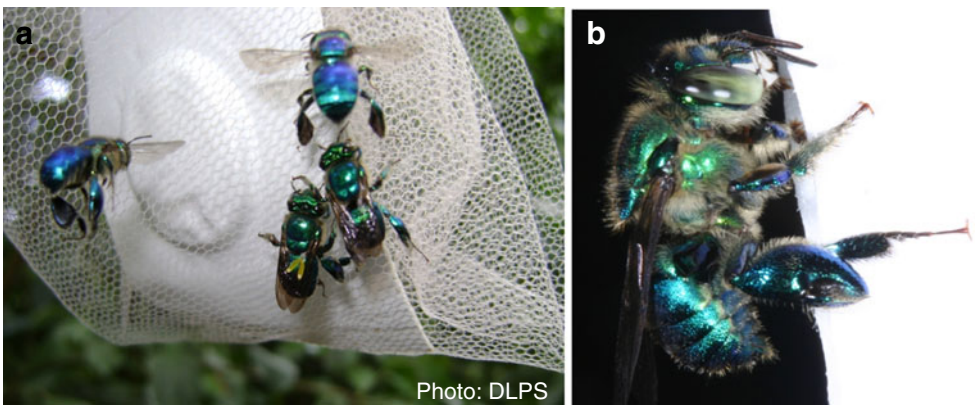


Fig. 1 **a** Male *Euglossa cyanura* (body length ~1.1 cm) arriving at a bubble cap bait, containing ipsdienol, near the Los Tuxtlas biological station, Veracruz, southern Mexico (one of the males is carrying a pollinarium of a *Gongora* orchid). **b** Close up of an ipsdienol-collecting male

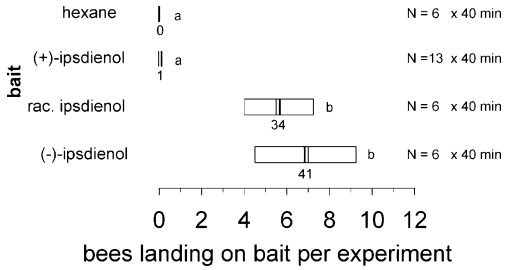


Fig. 2 Single-substance bioassays. Average number of male *Euglossa cyanura* landing on various ipsdienol and control (hexane) baits. Boxplots indicate the inter-quartile range (box), the median value (vertical line) and the arithmetic mean (bold vertical line). Numbers below the bold mean line represent the total number of bees. Treatment means followed by the same letters indicate those that are not different from each other (α=0.05)

enantioselectivity plays an important role in the fragrance collection behavior of male euglossines, which appear to have replaced endogenous pheromones with environmental odors (Zimmermann et al., 2006, 2009). To achieve pollination, a considerable number of orchid and other plant species of the neotropics take advantage of the attraction of euglossine males to volatile chemicals. Ipsdienol occurs in floral scents of orchids and aroids (Whitten et al., 1988; Schwerdtfeger et al., 2002; Knudsen et al., 2006), and it appears that it often occurs with its putative precursor, β-myrcene (Knudsen et al., 1999, 2006; Raguso, 2008). Unfortunately, it is not known whether or not (−)-ipsdienol is the predominant enantiomer in those neotropical plants (M. Whitten and J. Knudsen, personal communication). Other chiral compounds are attractive to male euglossines, and attraction may, in fact, be selective to one optical isomer. For example, of the 34 compounds listed by Roubik and Hanson (2004), 13 are chiral and, in at least one of them (α-pinene), attraction is mediated exclusively by one isomer [(−)-⟨-pinene] (Williams and Whitten, 1983). Another possible case of stereoselective attraction of male orchid bees is to hexahydrofarnesyl acetone, in which the *R,R*-isomer is more attractive than the racemate (Eltz et al., 2010).

In *Euglossa cyanura*, the observed preference to (−)-ipsdienol is associated with increased sensitivity of male antennae to this enantiomer. Previous EAG studies on orchid bees (Schiestl and Roubik, 2003; Eltz and Lunau, 2005; Eltz et al., 2006) found that the antennal chemosensory apparatus of euglossines responds to a broad range of compounds, in a rather generalized way; i.e., there was little evidence for component-specific antennal specialization. One notable exception is the antennal sensitivity of male *E. dilemma* to 2-hydroxy-6-nona-1,3-dienylbenzaldehyde, a high molecular weight compound that is both behaviorally attractive and present in large quantities in *E. dilemma* perfumes (Eltz et al., 2008). (−)-Ipsdienol is the first low molecular weight compound that has been found to elicit stronger antennal responses in males of an attracted species (*E. cyanura*) than in males of a non-attracted species (*E. mixta*). Thus, our findings strengthen the idea that antennal responses, to some extent, reflect behavioral preferences for certain odors by males. However, it remains unknown whether the increased antennal sensitivity of male *E. cyanura* is a cause or a consequence of (−)-ipsdienol preference. Both alternatives may apply but, perhaps, it is most parsimonious to assume that increased sensitivity

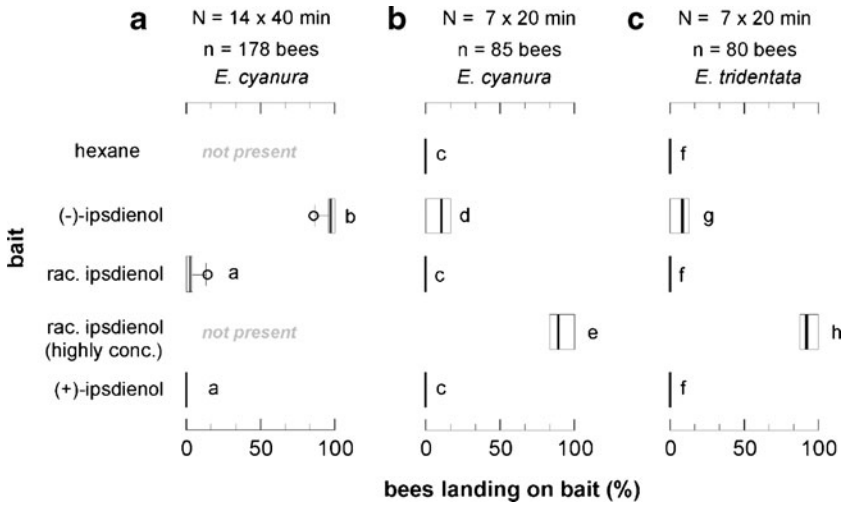


Fig. 3 Multiple-choice bioassays. Average percentages of male *Euglossa* species landing on various treatments. **a** *E. cyanura* on various bubble cap ipsdienol baits, all at the same concentration, plus a hexane control; **b** *E. cyanura* and **c** *E. tridentata* on the various bubble cap ipsdienol baits, all at the same concentration, plus a more concentrated racemate bait on filter paper, plus hexane control. Boxplots indicate the inter-quartile range (box), the median value (horizontal line), the arithmetic mean (bold line), 95% range (whiskers) and outliers. Treatments with the same letters indicate those that are different from each other (α=0.05)

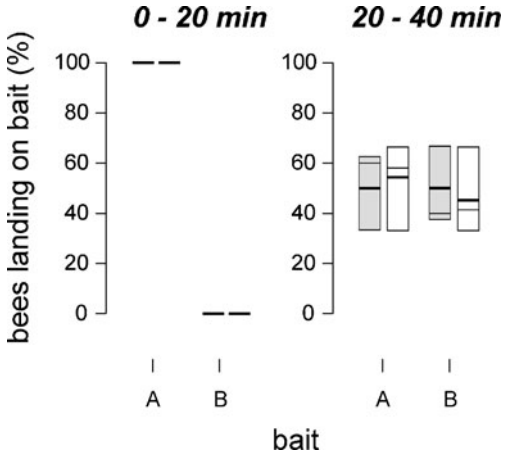


Fig. 4 Sequential bioassays. Test for intraspecific chemical cues. Percentages of bees landing on either of two baits (A, B). During the first 20-minute period ($N_{cyanura,\ 0–20\ min}$=7, N=46 bees; $N_{tridentata,\ 0–20\ min}$=7, N=38), bait A contained racemic ipsdienol and bait B hexane (control). During the second 20-minute period ($N_{cyanura,\ 20–40\ min}$=7, N=59 bees; $N_{tridentata,\ 20–40\ min}$=7, N=39), both baits had the same amount of ipsdienol, but bait A was distinguished from bait B by the presence of chemical traces left by odor-visiting bees during the first period. Boxplots (*Euglossa cyanura* = grey; *E. tridentata* = white) indicate the inter-quartile range (box), the median value (horizontal line), and the arithmetic mean (bold line). In the second period, there were no differences in landings to the two baits

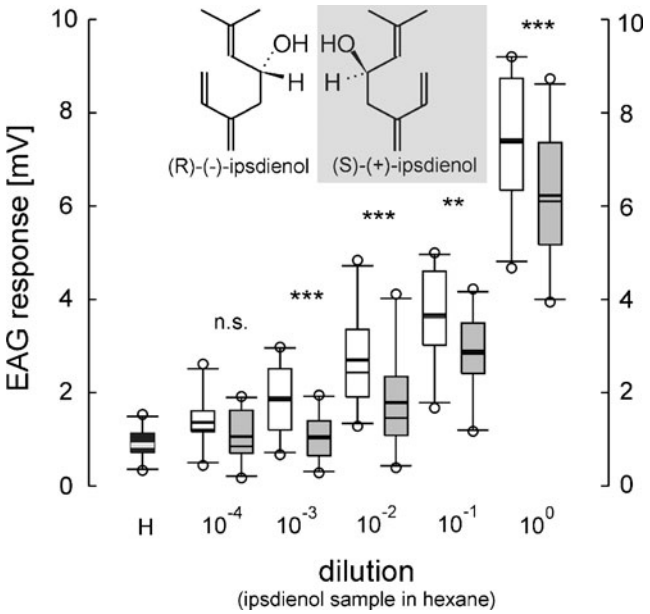


Fig. 5 Electroantennographic (EAG) responses of male *Euglossa cyanura* (N=10 bees) to different concentrations of the two enantiomers of ipsdienol. Boxplots indicate the inter-quartile range (box), the median value (horizontal line), the arithmetic mean (bold line), 95% range (whiskers) and outliers. Significant differences in responses to the two enantiomers are indicated by asterisks (*/**/***=P<0.05/0.01/0.001; n.s. = not significant = P>0.05). H = hexane (control). All responses to the enantiomers were larger than those to the hexane control (Paired T-tests; P<0.05), except for responses to (+)-ipsdienol at 10^{-4} and 10^{-3} dilutions (P>0.1)

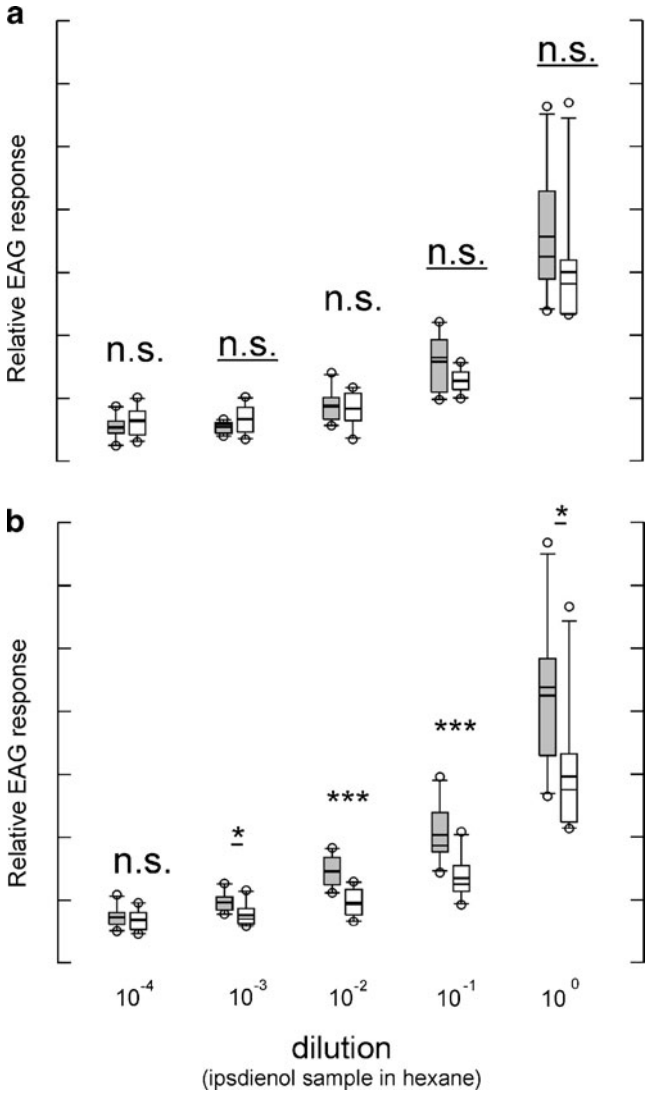


Fig. 6 Standardized electroantennographic (EAG) responses of male *Euglossa cyanura* (grey bars, N=10) and *E. mixta* (white bars, N=10) to different concentrations of (+)-ipsdienol (**a**) and (−)-ipsdienol (**b**). Boxplots indicate the inter-quartile range (box), the median value (horizontal line), the arithmetic mean (bold line), 95% range (whiskers), and outliers of the responses. Significant differences in standardized responses between the two species are indicated by asterisks (*/***=P<0.05/0.001; n.s. = not significant = P>0.05). The statistically relevant abbreviations are underlined when non-parametric statistics were applied

evolved as a consequence of an already existing behavioral preference. Ipsdienol is likely rare in the natural habitat, necessitating a highly responsive olfactory system to detect and locate a source. That male *E. cyanura* are highly responsive to this compound is supported by the observation that males were extremely quick to locate our baits. Often, it took the first males only 1–3 min to appear following bait exposure, surprising given the very low quantities of substance released from the bubble caps.

Whether or not male *E. cyanura* possess a class of (−)-ipsdienol-specific olfactory neurons on their antennae, as found in *Ips* bark beetles (Mustaparta et al., 1984), needs to be determined by single cell recording techniques. However, if they do possess such neurons, this could explain why they are not repelled by the presence of (+)-ipsdienol in an odor source. Males of both *E. cyanura* and *E. tridentata* preferred racemic ipsdienol whenever it was presented in concentrations greater than that of purified (−)-ipsdienol. Thus, it appears that it is the absolute amount of (−)-ipsdienol that matters, not whether (+)-ipsdienol is present or not; i.e., (+)-ipsdienol is neither a repellent nor an attractant. This would also explain why *E. cyanura* sometimes visited (+)-ipsdienol bubble caps containing trace proportions (3%) of (−)-ipsdienol during single-substance bioassays or after racemic and (−)-ipsdienol baits were removed. The behavioral indifference with regard to (+)-ipsdienol is surprising given that males perceive (+)-ipsdienol. The mechanisms by which males respond to small amounts of attractive (−)-ipsdienol, in mixtures with large amounts of (+)-ipsdienol, warrant further investigation. It is noteworthy that the situation is different for α-pinene, in which males are attracted only to the (−)-isomer. Here, the racemate is unattractive, suggesting that (+)-α-pinene acts as a repellent (Williams and Whitten, 1983).

No Signals and Cues Left on Visited Odor Sources In insects, there is mounting evidence for detection of chemical traces left by visitors to flowers and other resources (Schmidt et al., 2005; Wilms and Eltz, 2008; Goulson, 2009). Several species use these traces as cues when foraging to judge and detect the availability and profitability of particular odor sources. Since neither ipsdienol-collecting *E. tridentata* nor *E. cyanura* males preferred visited to unvisited ipsdienol baits, we conclude that these euglossines do not utilize such intraspecific chemical signals during odor collection. Presumably, odor originating from the resource alone represents sufficient information for males to detect the presence and profitability of an odor source. This is different to other resources like nectar and pollen advertised by entomophilous plants, in which odor quantity or quality do not necessarily match the actual amount of available resources; in such cases, it makes sense for the seekers to "watch out" for additional indicators of profitability. Whether euglossines utilize chemical cues left by previous flower visitors under such circumstances remains to be unveiled.

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the recent shift of the fly from its native host downy hawthorn, *Crataegus mollis* Sheele (Rosales: Rosaceae), to introduced Eurasian apple, *Malus domestica* Mill. (Rosales: Rosaceae) in the northeast United States about 160 years ago is often cited as an example of sympatric host race formation in action in the face of gene flow (Bush, 1966, 1969; Berlocher et al., 1993; Berlocher and Feder, 2002; Coyne and Orr, 2004; Smadja and Butlin, 2009). Genetic analysis (Feder et al., 1988; McPheron et al., 1988; Michel et al., 2010) and field studies (Feder et al., 1993, 1994) have confirmed the status of apple and hawthorn flies as partially ecologically reproductively isolated host races, the hypothesized first stage in sympatric speciation. Significant allele frequency, but not fixed, genetic differences have been documented for a number of loci between the apple and hawthorn fly races (Michel et al., 2010). In addition, mark-release-recapture studies in the field have shown that host fidelity while strong, is not absolute, and currently inter-host migration between apples and hawthorns occurs at a rate of ~4–6% per generation (Feder et al., 1994). Apple and hawthorn flies readily mate with each other in the lab and produce viable and fertile offspring (Dambroski et al., 2005). Consequently, there appears to be no obvious intrinsic post-zygotic reproductive isolation between the host races, and apple and hawthorn flies are primarily, if not exclusively, isolated by ecological barriers to gene flow.

Two host-related adaptations are important traits that facilitate the host shift of *R. pomonella* to apple and contributing to partial ecological reproductive isolation between the apple and hawthorn fly races. The first involves diapause life history adaptation matching the eclosion time of emerging adult flies to a difference in the fruiting times of apples vs. hawthorns (apple varieties favored by the fly generally bear fruit ~3–4 weeks earlier than hawthorns) (Bush, 1969; Feder et al., 1993, 1994). The difference in eclosion time generates allochronic mating isolation between the host races (Feder et al., 1993; Filchak et al., 2000). The second trait involves host-plant choice. Apple and hawthorn flies prefer to mate on or near the fruit of their respective host plants (Prokopy et al., 1971, 1972). Hence, differences in host plant choice translate directly to mate choice and generate premating reproductive isolation (Feder et al., 1994). One such difference involves volatile compounds emitted by ripening host fruit that are used to recognize and distinguish among alternative host plants (Linn et al., 2003; Forbes et al., 2005). Not only do apple and downy hawthorn flies preferentially orient to their respective natal fruit volatiles, but their upwind-directed flight also is antagonized (arrested) by non-natal volatiles (Forbes et al., 2005; Linn et al., 2005a). Antagonism is important because it indicates that when flies shift to a new host plant, not only do they rapidly evolve and positively behaviorally respond to the volatile profile of the new fruit (where offspring survivorship is higher) but also avoid certain volatiles of the ancestral host fruit (where fitness is lower).

Divergence along these two axes: eclosion phenology and host odor response, has led to considerable, but still incomplete reproductive isolation between races. Mark-recapture studies estimate a migration rate of ~4–6% at sympatric sites (Feder et al., 1994), more than enough to homogenize allele frequencies of the races in the absence of selection (Feder, 1998). While F_1 hybrids between races are intrinsically viable (Reissig and Smith, 1978), they are ecologically unfit due to life history tradeoffs (Filchak et al., 2000) and diminished olfactory response to either parental host fruit (Linn et al., 2004). Thus, genetic differences between races (Feder et al., 1988; McPheron et al., 1988; Michel et al., 2010) are actively maintained by divergent selection on these two key ecological traits.

In contrast to the northern USA, *R. pomonella* infests an array of different hawthorn species in the South (Note: domesticated apple does not thrive in the southern USA, and so the apple race is limited in its distribution to the Northeast, Midwest, and Appalachian Mountains). In this regard, it has been suggested that perhaps the apple race did not originate via a sympatric host shift from the endemic downy hawthorn in the Northeast but from migration of a geographically differentiated, hawthorn-infesting form of the fly from the South (Carson, 1989; although see Bush et al., 1989). This would imply that in the southern USA, a "pre-adapted" hawthorn-infesting population of *R. pomonella* exists that preferentially responds to apple fruit volatiles and is antagonized by downy hawthorn fruit volatiles. It also is possible, however, that *R. pomonella* has formed a series of sympatric host races on the various species of native hawthorn present in the South (Berlocher and Enquist, 1993). Indeed, the five major hawthorn hosts of *R. pomonella* in the South (western mayhaw, *C. opaca*; eastern mayhaw, *C. aestivalis*; blueberry hawthorn, *C. brachyacantha*; green hawthorn, *C. viridis*; and downy hawthorn, *C. mollis*) generally overlap in their geographic ranges, but differ in key traits such as fruiting time, host fruit color and size, and potentially fruit volatiles, thus presenting the opportunity for host-related divergence. In this case, the southern hawthorn races would represent an example of a natural sympatric radiation onto a suite of native host species, complimenting the documented historical shift of the fly onto domesticated apple in recent times.

To investigate the possibilities of preformed apple vs. native hawthorn host races in the South, we initiated studies of the fruit volatile profiles and behavioral responses of *R. pomonella* to the major hawthorn hosts in the southern USA. Here, we focus on three related *Crataegus* species in the series *Aestivales* collectively known under the common name of "mayhaw". *Rhagoletis pomonella* infests both a western (*C. opaca*) and eastern (*C. aestivalis*) distributed

species of mayhaw in the South. The range of *C. opaca* extends from eastern Texas, through all of Louisiana to southwestern Mississippi, with a northern limit in extreme southern Arkansas (Fig. 1). *Crataegus aestivalis* is found from southern Alabama through southern Georgia and central Florida, as well as along the Atlantic coast to North Carolina. Both *C. aestivalis* and *C. opaca* are grown commercially in their respective regions; the fruit is used to make jelly, syrup, and wine. The two species have an early fruiting phenology (mid April to late May), large edible fruits, and a requirement for damp and often flooded soil. The mayhaws are distinguished morphologically by the eastern *C. aestivalis* in having more glabrous oblong-ovate leaves, as opposed to the more cuneate leaves of the western *C. opaca* (Sargent, 1933). The fruit of *C. aestivalis* is smaller and more oblong in shape than *C. opaca*, which has a more depressed, globose fruit. A third described species, *C. rufula*, occurs primarily at the intersection of the eastern and western species' ranges between the lower Pearl River and Pascagoula River drainages in Mississippi. It is intermediate between *C. opaca* and *C. aestivalis* in both fruit and leaf morphology, and may represent a hybrid species. Collection of ripe *C. rufula* fruit from four field sites in this region of Mississippi from 2008 to 2010 failed to yield any *Rhagoletis* infestation.

The distinctive early fruiting time of southern mayhaws makes them prime candidates for the source of a potential shift to apple in the North. To assess this possibility, we developed volatile blends for the three mayhaw species through a combination of gas chromatography/electroantennographic detection (GC-EAD) and gas chromatography/mass spectrometric (GC-MS) analysis of both solid phase microextraction (SPME) and adsorbent samples collected from fruit headspace. Behavioral flight tunnel assays also were conducted using the different adsorbent extracts, mayhaw blends, and our previously developed apple, downy hawthorn, and flowering dogwood (*Cornus florida*) blends to test for possible olfactory pre-adaptation of mayhaw flies to apple. We report results showing that while mayhaws share some fruit volatiles in common with apple, the fruit odor profiles also differ in several key volatiles. Moreover, while eastern and western mayhaw flies preferentially oriented to their respective natal mayhaw blends, they were less responsive to the apple and downy hawthorn blends. Our findings are, therefore, consistent with the possibility of southern USA hawthorn-infesting host races of *R. pomonella*.

Methods and Materials

Insects Flies were collected, from a total of four different mayhaw sites (Fig. 1; Table 1), as larvae in infested host fruit and reared to adulthood in the Notre Dame laboratory

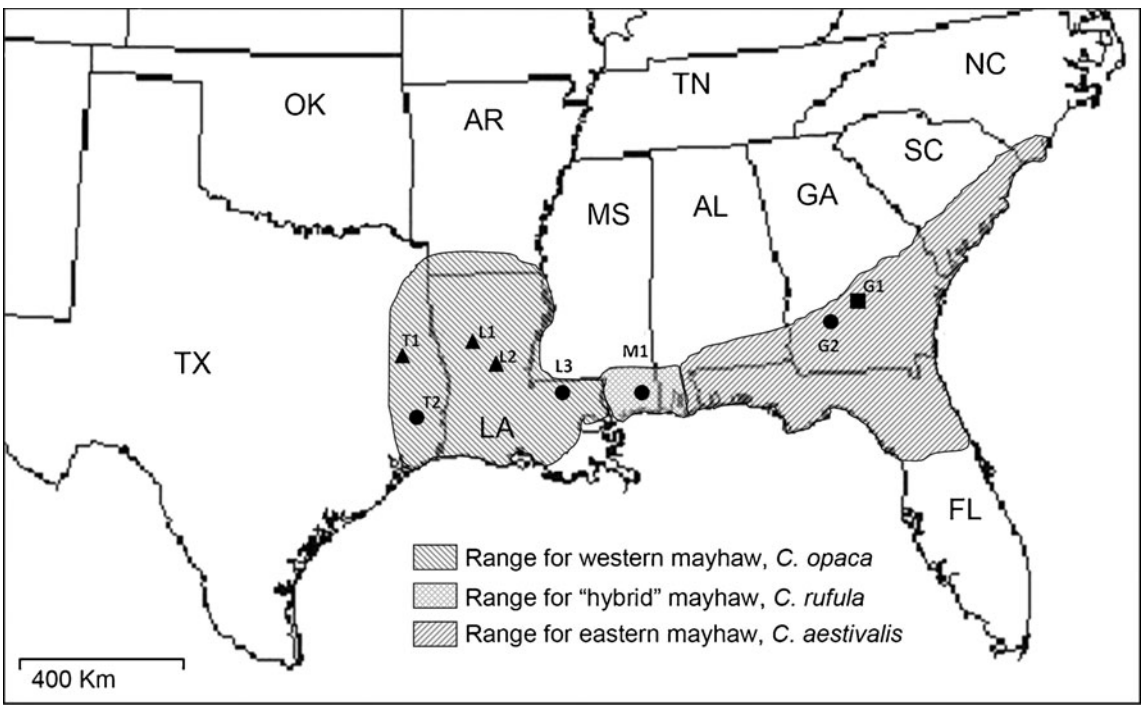


Fig. 1 Range map for western, *Crataegus opaca*, eastern, *C. aestivalis,* and "hybrid", *C. rufula*, mayhaws with collection sites for fruit (circles), flies (triangles), or both (squares). Abbreviations for each of the States indicated are: *AL* Alabama, *AR* Arkansas, *FL* Florida, *GA* Georgia, *LA* Louisiana, *MS* Mississippi, *NC* North Carolina, *OK* Oklahoma, *SC* South Carolina, *TN* Tennessee, *TX* Texas

Table 1 Collection sites for mayhaw fruits (*Crataegus*) and flies and their coordinates as marked in Fig. 1

Map name	Host plant species	Location	Latitude	Longitude
G1	*C. aestivalis*	Byron USDA-ARS, Peach Co, GA	32º 39' N	83º 43' W
G2	*C. aestivalis*	Arabi Warwick Rd, Crisp Co, GA	31º 50' N	83º 47' W
L1	*C. opaca*	Lake Iatt, Grant Parish, LA	31º 38' N	92º 38' W
L2	*C. opaca*	Dewey Wills WMA, La Salle Parish, LA	31º 27' N	92º 06' W
T1	*C. opaca*	SFA exp. forest, Nacogdoches, Co, TX	31º 30' N	94º 46' W
T2	*C. opaca*	HW Jackson Farm, Polk Co. TX	30º 31' N	94º 42' W
L3	*C. opaca*	LSU Idlewild, East Feliciana Parish, LA	30º 49' N	90º 57' W
M1	*C. rufula*	Parker Lake, Jackson Co. MS	30º 38' N	88º 35' W

using standard *R. pomonella* husbandry techniques (Neilson, 1965; Dambroski and Feder, 2007). Adult flies were shipped to the Cornell lab and kept in a walk-in environmental chamber at 23-24°C, 16 L:8D photoperiod, and 65–70% r.h., on an artificial diet made of water, sugar, vitamins, casein hydrolysate, and salt mixture prior to testing (Fein et al., 1982). Adult flies at 0–7 and 10–21-d-old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively. Younger flies were used for the GC-EAD analyses because they are more robust and stable in the EAD apparatus than older flies (Nojima et al., 2003a). In contrast, *R. pomonella* flies do not respond behaviorally to volatiles until they reach reproductive maturity at > 8d post-eclosion (Fein et al., 1982).

Fruit Western mayhaw (*C. opaca*) fruit came from two locations: the Louisiana State University Idlewild Experimental Field Station, East Feliciana Parish, LA, USA, and the H.W. Jackson Farm, Polk Co., TX, USA (Fig. 1; Table 1). Eastern mayhaw (*C. aestivalis*) fruit also came from two sites: the USDA southern fruit and nut station, Byron, GA, USA, and Crisp Co., GA, USA (Fig. 1; Table 1). Fruit from the morphologically intermediate *C. rufula* were collected from Parker Lake in the Pascagoula Wildlife Management Area, Jackson Co., MS, USA (Fig. 1; Table 1). Fruit were collected from off of trees in the field and shipped overnight to the Cornell lab for volatile characterization. While it is possible that fruit on trees emit a different blend than newly picked fruit, this is an inherent methodological constraint of volatile collection. In this regard, we note three points. First, our goal was not to directly determine the exact volatile profile of fruit on trees, but rather to identify the key behaviorally active compounds inducing fly response, and to develop synthetic blends that elicit levels of orientation at least as high as whole fruit extracts. Candidate compounds were identified by GC-EAD analysis. Thus, while the composition of eluded compounds from newly picked fruit may differ from unpicked fruit on trees, the key volatiles for behavior should still be present and identified by GC-EAD. Second, previous studies have shown that *Rhagoletis* flies do not respond to all the volatiles emitted by fruit but a crucial subset of compounds (Zhang et al., 1999; Nojima et al., 2003a, b; Linn et al., 2003). Thus, determination of the exact composition of volatiles emitted by fruit on trees is not as important as identification of the subset of key compounds. Third, we eluted volatiles from activated charcoal adsorbent filters in collecting chambers every 24 h for a given fruit sample, and we combined the elution across 5 collecting days, as well as performing SPME sampling on the day following fruit collection. Thus, we sampled volatiles encompassing a period as close as we could following fruit collection in a manner to best ensure that all compounds were sampled in sufficient quantity to be detected by GC-EAD analysis.

Adsorbent Sampling Headspace volatiles were collected from whole fruit by using 2.4 l closed volatile collection chambers (ARS, Inc., Gainesville, FL, USA; glass shop, Cornell University, Ithaca, NY, USA) with one air inlet adapter (7 mm ID) on the top and an outlet adapter (7 mm ID) on the bottom wall. Field collected fruit (500–800 ml in volume) were cleaned gently with distilled water, thoroughly dried, and then immediately put into a collection chamber. Clean air from a filtering and delivery system (ARS Inc., Gainesville, FL, USA) was pushed into the chamber at 0.7 L min^{-1} through the top inlet port, and volatiles from fruit were pushed out through volatile traps (activated charcoal adsorbent filters, ORBO32-small, Supelco Inc., Bellefonte, PA, USA) on the bottom of the chamber. An additional ORBO filter was used to ensure that no breakthrough occurred. For each sample of fruit, adsorbent collections were made over a 5 d period at room temperature using the same volatile trap. For each new fruit sample, the chambers were washed with acetone, purged with clean filtered air for 24 h, and new ORBO filters were put in place. Volatiles were eluted with 500 µl methylene chloride every 24 h from the filters of a given fruit sample and combined across the 5 collecting days. The combined extract was kept at −20°C and subjected to GC-EAD, GC-MS, and flight tunnel analyses.

SPME Sampling A glass jar (500 ml) with Teflon liner screwcap (Wheaton, Milliville, NJ, USA), containing ca. 150 g of fruit, was used for SPME sampling. A carboxen–polydimethylsiloxane-coated SPME fiber (film thickness 85 μm, Supelco) was conditioned in the GC injector (280°C) for 5 min, and then passed through the small hole on the cap into the headspace of the jar. After a 10 to 20-min exposure, collected volatiles on the fiber were immediately subjected to GC-EAD analysis. The sampling container was washed with acetone before sampling new fruit.

Coupled Gas Chromatographic-Electroantennographic Detection (GC-EAD) Analysis Coupled GC-EAD analysis was performed as described previously (Zhang et al., 1999; Nojima et al., 2003a,b; Cha et al., 2008) using a Hewlett Packard 5890 Series II gas chromatograph equipped with a non-polar EC-1 capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; Alltech Associates, Inc., Deerfield, IL, USA) or a Shimadzu GC-17A gas chromatograph equipped with a polar EC-Wax Econo-Cap capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; Alltech) in the splitless mode. The oven temperature was programmed at 40°C for 5 min, then increased by 15°C/min to 250°C, and held for 5 min. Injector and detector temperatures were set at 280°C and 270°C, respectively. Nitrogen gas was the carrier at a flow of 2 ml/min. The column effluent was split 1:1 in the oven via a "Y" glass splitter (Supelco). One arm of the splitter led to the flame ionization detector (FID) (270°C) and the other to the heated EAD port (270°C).

Whole head preparations were made of individual flies for GC-EAD analysis (Nojima et al., 2003a). The head was separated from the body and its antennae positioned between two gold wire electrodes immersed in saline-filled (*Drosophila* ringer solution; 46 mmol NaCl, 182 mmol KCl, 3 mmol $CaCl_2$, and 10 mmol Tris HCl at pH 7.2) micropipettes in an acrylic holder. The antennal holder was placed inside a humidified condenser and maintained at 5°C. The output signal from the antenna was amplified by a customized high input impedance DC amplifier and recorded on an HP 3390A integrator.

Chemical Analysis GC-MS was carried out as previously described (Zhang et al., 1999; Nojima et al., 2003a,b; Cha et al., 2008) with a Shimadzu GCMS-QP5050A quadrupole mass spectrometer in EI (at 70 eV) scan mode coupled with a Shimadzu GC-17A equipped with a nonpolar DB-1 ms capillary column or a polar EC-Wax Econo-Cap capillary column. Helium was the carrier gas (54 kPa at 1.1 ml/min). GC conditions and temperature program were as for the GC-EAD analyses. The interface temperature was set at 260°C. Volatile compounds were identified by comparison of chromatographic retention times and mass spectra with those of authentic standards analyzed on the same instrument. The identification was further verified by antennal responses in the GC-EAD analyses to the standard compounds. Quantification of the relative ratio of the compounds that elicited EAD responses was made from the adsorbent collection based on total ion abundances from GC/MS analyses. As we did not add an internal standard to adsorbent samples for behavioral testing purposes, standard solutions containing 0.1, 1, 10, and 100 ng of the each compound that elicited EAD responses in 1 μl of methylene chloride were prepared and analyzed to obtain calibration curves.

Chemicals The compounds pentyl hexanoate, hexyl butanoate, butyl hexanoate, propyl hexanoate, pentyl butanoate, butyl butanoate, 1-octen-3-ol, 3-methylbutan-1-ol, 3-methylbutyl acetate, dimethyl trisulfide, β-caryophyllene (purities>98%), and dihydro-β-ionone (> 90%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Butyl acetate (> 99%) and pentyl acetate (> 98%) were purchased from TCI America (Portland, OR, USA), and ethyl acetate (99.9%) was purchased from Fischer Scientific (Pittsburgh, PA, USA). The compound (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was synthesized by oxidation of geraniol and then by Wittig reaction with methylenetriphenylphosphorane (Greenwald et al., 1963) and purified (> 97% with> 97% *E*-isomer by GC/MS) using flash chromatography on silica gel. The synthesis product was eluted with hexane and then subjected to Kugelrohr distillation.

Synthetic Blends and Flight Tunnel Sources We prepared synthetic blends for western, eastern, and hybrid mayhaw based on the EAD active volatile compounds identified from GC-EAD and GC-MS analyses (Fig. 2). The synthetic blends for apple, downy hawthorn, and flowering dogwood were based on Zhang et al. (1999) and Nojima et al. (2003a, b). Table 2 shows the proportion of each compound in the respective blends. The western mayhaw, eastern mayhaw, hybrid mayhaw, and apple blends were prepared such that the concentration of the total volatile mixture was 1 μg/μl in hexane. Downy hawthorn and flowering dogwood blends were prepared as described in Nojima et al. (2003a,b). As in our previous flight tunnel studies, we applied 200 μl of a synthetic blend to a red rubber septum (hexane-washed; Thomas Scientific, Swedesboro, NJ, Cat. No. 1780 J07) as the corresponding doses elicited maximal levels of upwind flight (Zhang et al., 1999; Linn et al., 2003; Nojima et al., 2003a,b). The septum sources were prepared 60 min prior to a test and were clipped onto the bottom of a 7.5 cm red plastic sphere (Gempler's Inc., Mt. Horeb, WI, USA). Fresh sources and red spheres were used for each replicate. For adsorbent extracts, we adjusted (either by diluting with hexane or concentrated under a gentle stream of nitrogen gas) the concentration (GC peak

size) of GC-EAD active volatiles to match the GC profiles of the synthetic blend at 1 μg/μl concentration, and applied 200 μl of the extract to a rubber septum.

Modified Synthetic Blends Based on the similar high response levels of flies to whole fruit adsorbent extracts and synthetic blends (see next section below), we prepared three modified blends by adding and/or subtracting compounds from the complete blends, to determine the relative importance of individual volatiles in the blend, or those that might function as inter-specific behavioral antagonists (Table 3). Because of the limited availability of flies, we concentrated our effort on the compounds butyl acetate, found in the eastern mayhaw blend but not in the western blend, and 3-methylbutan-1-ol, previously demonstrated to be an essential volatile for the upwind flight response of northern downy hawthorn flies (Linn et al., 2005b) and functioning as a behavioral antagonist for apple-origin flies. Blend W1 represented the complete 6-component western blend (Wc) with butyl acetate added. Blends E1 and E2 represented the complete 8-component eastern blend (Ec) with butyl acetate removed, and with both butyl acetate and 3-methylbutan-1-ol removed, respectively. The modified blends were prepared so that each compound was always in the same amount as in the corresponding complete blends, thus eliminating the potential effect of variation in concentration. For flight tunnel testing, we applied 200 μl of the modified blends to a rubber septum. The concentration of each modified blend is listed in Table 3.

Flight Tunnel The response of flies to host fruit volatiles was measured in a sustained-flight tunnel (Nojima et al., 2003a). Flight-tunnel conditions were 23-24°C, 50-70% r.h., 35 cm/s wind speed, 1500 lux light intensity. Sexually mature adult flies (males and females) 10-21-d-old post-eclosion that had never been previously exposed to the fruit volatile blends (i.e., fruit odor-naïve flies) were tested during the 3rd to 11th h of the 16 h photophase period in the flight tunnel. Flies were selected from holding cages located in a separate, environmentally controlled room, placed singly in glass vials, taken to the room housing the flight-tunnel, and then allowed to acclimate for at least 30 min before testing. Individual flies were transferred to a screen holding cage, which was then placed on a release stand such that the open end of the cage faced upwind at a distance of 1 m to the odor source. Flies were given 1 min to respond, and scored by a human observer for the following behaviors: 1) remain in release cage = fly did not take flight, but remained in the release cage, generally walking and grooming; 2) taking flight = flight from the release cage to the sides, top or floor of the tunnel; and 3) upwind-directed flight = fly faced upwind, walked to the edge of the release cage (100 cm from source), took flight and initiated upwind oriented flight in the odor plume. An upwind flight response was scored as positive if the fly exhibited plume-oriented flight for at least 50 cm toward the sphere [we previously showed that at this distance flies are not attracted to the red sphere alone with no odor source (Linn et al., 2003)]. In some cases, flies made complete flights to the red sphere, but mostly their upwind oriented flights ended within 20–25 cm of the sphere with the fly landing on the floor or side walls of the tunnel. We suspect this was due to negative visual effects of the red sphere at close range. Large red spheres simulate an apple fruit, or cluster of dogwood and red hawthorn (*C. mollis*) fruit, but may not be an optimal visual stimulus for mayhaw flies. The red sphere was retained in the present study to provide a consistent assay environment between the current experiments and previous studies (Linn et al., 2003). Also, in the following sections, reference to 'agonist' volatiles refers to those that contributed to the positive upwind directed flight response to a particular blend, and 'antagonist' refers to a non-natal host volatile that, when added to a blend resulted in a significant reduction in upwind directed flights. The effect of the antagonist was characterized by flies exhibiting arrested flight within 25 cm of the release cage, hovering (1–2 s) in the plume, followed by cross-wind casting, then turning and flying in a downwind direction and landing on the floor or side walls of the tunnel. These behaviors were strikingly similar to the pattern observed for behavioral antagonists in moth sex pheromone blends (Linn and Roelofs, 1995; Cardé and Haynes, 2004).

For each of the host plants, our experimental protocol involved first testing adsorbent extracts in the flight tunnel, with the expectation that response levels should be high, indicating that the behaviorally active blend was contained in the extract, and that no potential deterrent compounds were present, such as might occur with over-ripe or rotting fruit. Given a high response level to the extract, we then tested the candidate synthetic blends that were selected from the GC-EAD profiles based on the consistency of the responses over the majority (>75%) of the profiles. Again, the expectation was that response levels should be high and equivalent to those observed with the extracts. We then tested flies to modified blends that had volatiles subtracted or added to the complete blends to determine whether all of the compounds identified from the GC-EAD profiles were required for maximal response (upwind directed flight). For each extract or synthetic blend, different sets of flies were

Fig. 2 Simultaneous recorded GC-EAD responses of mayhaw-origin fly antennae to corresponding adsorbent collections. Different traces indicate antennal response of **a** western mayhaw-origin flies to western adsorbent samples, **b** eastern mayhaw-origin flies to eastern adsorbent samples, and **c** western mayhaw-origin flies to "hybrid" adsorbent samples

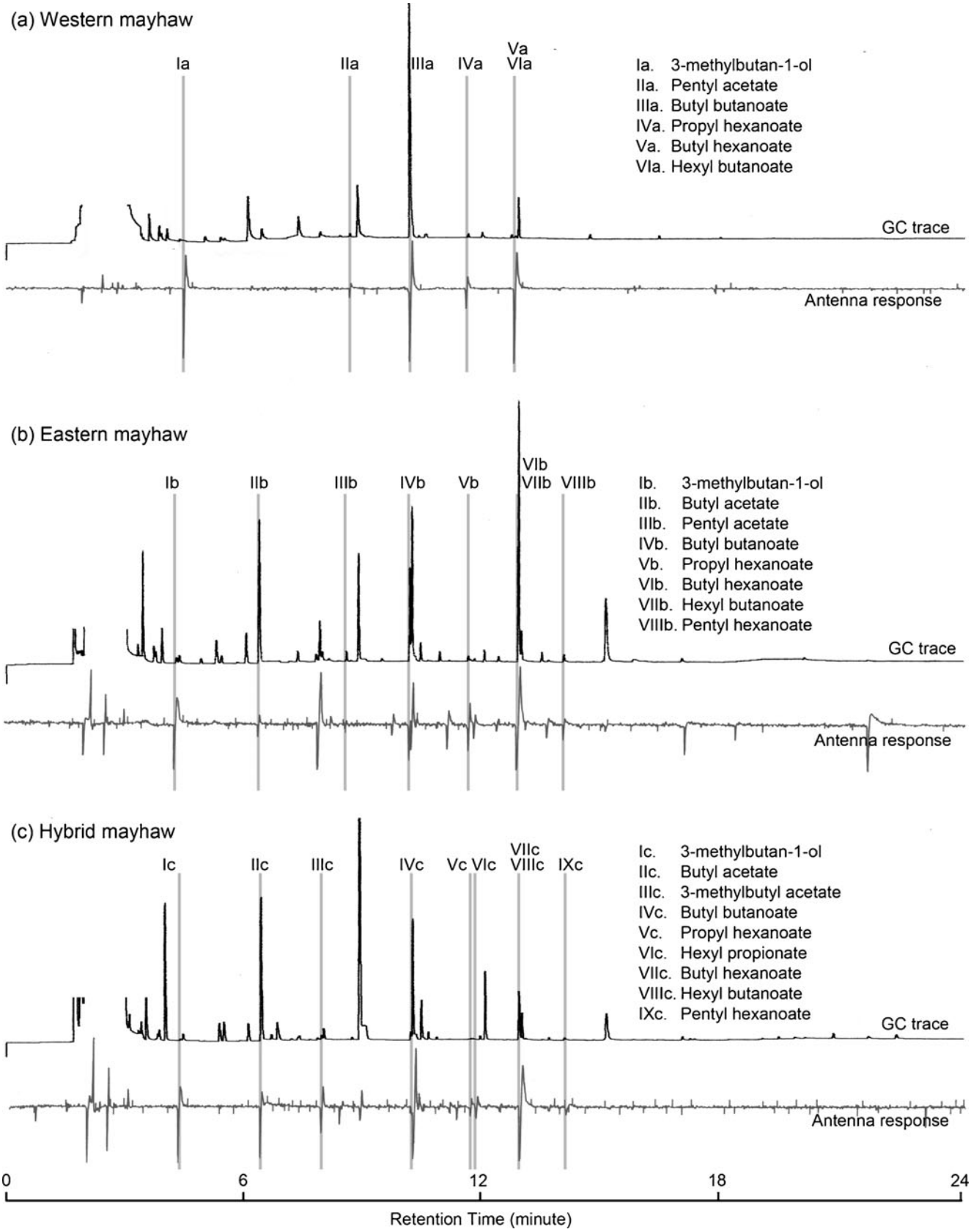

(a) Western mayhaw
Ia
IIa
IIIa
IVa
Va
VIa
Ia. 3-methylbutan-1-ol
IIa. Pentyl acetate
IIIa. Butyl butanoate
IVa. Propyl hexanoate
Va. Butyl hexanoate
VIa. Hexyl butanoate
GC trace
Antenna response
(b) Eastern mayhaw
Ib
IIb
IIIb
IVb
Vb
VIb
VIIb
VIIIb
Ib. 3-methylbutan-1-ol
IIb. Butyl acetate
IIIb. Pentyl acetate
IVb. Butyl butanoate
Vb. Propyl hexanoate
VIb. Butyl hexanoate
VIIb. Hexyl butanoate
VIIIb. Pentyl hexanoate
GC trace
Antenna response
(c) Hybrid mayhaw
Ic
IIc
IIIc
IVc
Vc
VIc
VIIc
VIIIc
IXc
Ic. 3-methylbutan-1-ol
IIc. Butyl acetate
IIIc. 3-methylbutyl acetate
IVc. Butyl butanoate
Vc. Propyl hexanoate
VIc. Hexyl propionate
VIIc. Butyl hexanoate
VIIIc. Hexyl butanoate
IXc. Pentyl hexanoate
GC trace
Antenna response
0
6
12
18
24
Retention Time (minute)

Table 2 The relative ratios (%) of volatile compounds in different synthetic blends. Ratios for three mayhaw blends were based on GC/MS analyses of volatile adsorbent samples collected from 3 different mayhaw species. Ratios for apple, downy hawthorn and dogwood are those for the synthetic blends previously developed by Zhang et al. (1999) and Nojima et al. (2003a, b). DMNT indicates (*3E*)-4,8-dimethyl-1,3,7-nonatriene

Chemicals	Synthetic blends (%)					
	Western mayhaw (Wc)	Eastern mayhaw (Ec)	"Hybrid" mayhaw (Hc)	Apple	Downy Hawthorn	Flowering Dogwood
3-methylbutan-1-ol	44	2	1		4	27.5
Butyl acetate		47	57			
Pentyl acetate	6	2				
Butyl butanoate	6	12	5	10		
Propyl hexanoate	6	1	1	4		
Butyl hexanoate	26	25	23	37	0.01	
Hexyl butanoate	12	9	6	44		
Pentyl hexanoate		2	3	5		
3-methylbutyl acetate			3		1.5	0.9
Hexyl propionate			1			
Ethyl acetate					94.3	54.9
DMNT					0.07	
Dihydro-β-ionone					0.1	
Dimethyl trisulfide						1.9
1-octen-3-ol						9.1
β-caryophyllene						5.8

tested as the flies became available from field collections of infested fruit. For each set of experiments, almost all of the flies were tested to all of the treatments, usually over a continuous period of 2–4 d, with no more than 3 treatments being tested on a given day. In previous studies, we have shown that *R. pomonella* flies can be repeatedly tested without affecting odor preference as displayed in the flight tunnel (Dambroski et al., 2005). Differences in the frequency of upwind flight were compared using Fisher's Exact Test in R (R development core team, Vienna, Austria).

Table 3 Relative ratio (%) of volatile compounds in the complete western mayhaw (Wc) and eastern mayhaw (Ec) synthetic blends and their modified blends (W1, E1, and E2). We prepared the modified blends so that the same compound was always in the same amount as in corresponding complete blends, thus eliminating the potential effect of variation in concentration. For flight tunnel tests, 200 μl of Wc or Ec (prepared at 1.0 μg/μl), thus 200 μg of total volatile compounds, were loaded onto a rubber septum. The ratio and concentration of modified blends (W1, E1, and E2) were formulated as found in Wc or Ec. For flight tunnel tests of the modified blends, we also loaded 200 μl of W1, E1, or E2 to a rubber septum. Bold characters indicate the modified compounds

Chemicals	Synthetic blends (%)				
	Western mayhaw (Wc)	Modified Western mayhaw (W1)	Eastern mayhaw (Ec)	Modified Eastern mayhaw (E1)	Modified Eastern mayhaw (E2)
3-methylbutan-1-ol	**44**	**44**	**2**	**2**	
Butyl acetate		**47**	**47**		
Pentyl acetate	6	6	2	2	2
Butyl butanoate	6	6	12	12	12
Propyl hexanoate	6	6	1	1	1
Butyl hexanoate	26	26	25	25	25
Hexyl butanoate	12	12	9	9	9
Pentyl hexanoate			2	2	2
Total%	100	147	100	53	51
Concentration (μg/μl)	1.0	1.47	1.0	0.53	0.51

Results

Identification of Volatiles from Western, Eastern, and C. rufula Mayhaw Fruit Mayhaw fruit was analyzed using SPME and adsorbent extracts with GC-EAD and GC-MS. Figure 2 a-c shows GC-EAD recordings from the antennae of a fly from each mayhaw-origin population exposed to the whole fruit extract at a stage when >90% of the fruit was ripe. We note that whereas the traces indicate the presence of some unidentified peaks with EAD responses, these responses were not present in the majority (>75%) of GC-EAD runs examined. The relative ratio of compounds that elicited EAD responses from flies in each geographic location estimated with GC-MS are listed in Table 2.

For western mayhaw (*C. opaca*), fruit was analyzed from two samples collected in 2007 (Idlewild, East Feliciana Parish, LA, USA, and Polk Co., TX, USA). A total of 12 different antennal pairs of 9 female and 3 male flies (1–10 replicate runs/pair) were used for the GC-EAD analysis. All of the flies were of western mayhaw-origin from Dewey Wills, LaSalle Parrish, LA, USA. Compounds that consistently elicited EAD responses were identified as (**Ia**) 3-methylbutan-1-ol, (**IIa**) pentyl acetate, (**IIIa**) butyl butanoate, (**IVa**) propyl hexanoate, **(Va)** butyl hexanoate, and (**VIa**) hexyl butanoate. Two adsorbent extracts of volatiles from whole mayhaw fruit from each location that were made for quantitative analysis of key volatiles (GC-MS) and flight tunnel analysis were identical.

For eastern mayhaw (*C. aestivalis*), fruit collected in 2007 from Byron, GA, USA and in 2010 from Crisp Co., GA, USA, was analyzed. A total of 7 different antennal pairs of 5 female and 2 male flies (all from Byron; 1–10 replicate runs/pair) were used for the GC-EAD analysis. The corresponding compounds that consistently generated EAD responses from tested flies were identified as (**Ib**) 3-methylbutan-1-ol, (**IIb**) butyl acetate, (**IIIb**) pentyl acetate, (**IVb**) butyl butanoate, (**Vb**) propyl hexanoate, **(VIb)**, butyl hexanoate, (**VIIb**) hexyl butanoate, and (**VIIIb**) pentyl hexanoate.

Fruit from the potential hybrid mayhaw, *C. rufula*, was analyzed from a 2010 collection made at Parker Lake, MS, USA. A total of 7 different antennal pairs of 5 female and 2 male eastern mayhaw-origin flies (1–10 replicate runs/pair) from Byron, GA, USA, were used for the GC-EAD analysis. The corresponding compounds that consistently generated EAD responses from tested flies were identified as (**Ic**) 3-methylbutan-1-ol, (**IIc**) butyl acetate, (**IIIc**) 3-methylbutyl acetate (**IVc**) butyl butanoate, (**Vc**) propyl hexanoate, **(VIc)** hexyl propionate, (**VIIc**) butyl hexanoate, (**VIIIc**) hexyl butanoate, and (**IXc**) pentyl hexanoate.

Behavioral Responses of Mayhaw-Origin Flies to Natal Adsorbent Extracts and Non-natal Northern Volatile Blends Western and eastern-origin flies were tested first in the flight tunnel to western, eastern, and *C. rufula* adsorbent extracts and to the previously developed apple, northern downy hawthorn (DH), and flowering dogwood (DW) blends (Fig. 3). A total of 30 western flies from *C. opaca* fruit collected from La Salle Parish, (N=17) and Grant Parish (N=13), LA, USA, were tested to the western (W-MH) and eastern mayhaw (E-MH) adsorbent extracts, and 13 of the western mayhaw flies from La Salle Parish also were tested to the extract of the hybrid (H-MH) (Fig. 3a). A higher proportion of western flies displayed upwind flight to the western extract (67%) than to the eastern extract (10%; P<0.001, 1 *df*), hybrid extract (0%; P<0.001, 1 *df*), apple blend (3%; P<0.001, 1 *df*), northern downy hawthorn blend (30%; P=0.009, 1 *df*), and flowering dogwood blend (0%; P<0.001, 1 *df*).

For eastern mayhaw, a total of 27 flies from *C. aestivalis* fruit collected from Byron, GA, USA, were tested (Fig. 3b).

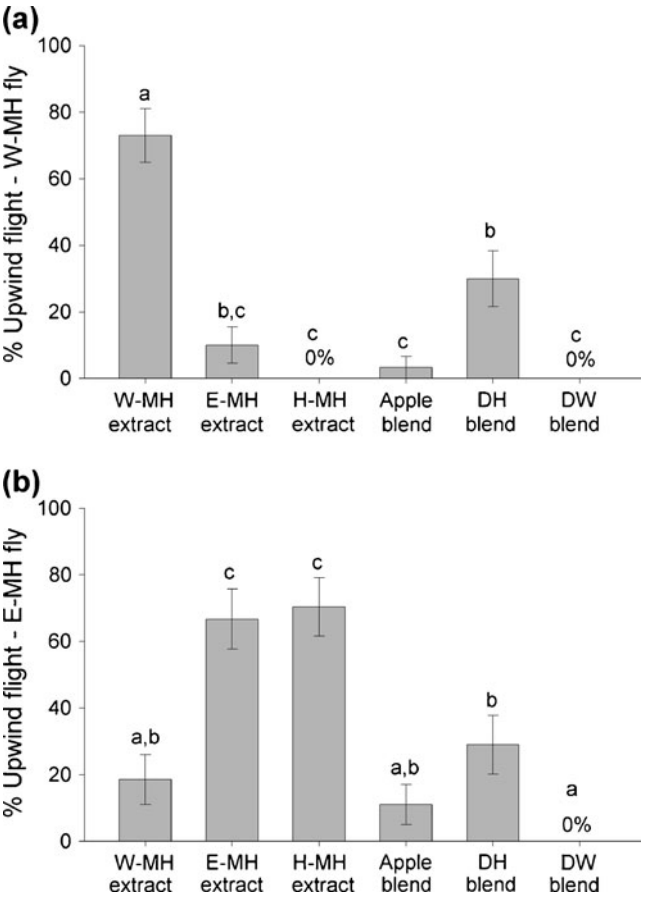


Fig. 3 Upwind flight responses (%±1 *SE*) of **a** western mayhaw-origin flies and **b** eastern mayhaw-origin flies to western, eastern and hybrid mayhaw whole fruit adsorbent extracts and previously developed volatile blends for 3 northern host plants: apple, downy hawthorn, and flowering dogwood. List of abbreviations used and what they stand for: W-MH, western mayhaw; E-MH, eastern mayhaw; H-MH, hybrid mayhaw; DH, downy hawthorn; DW, flowering dogwood. Different letters on bars indicate significant differences (P<0.05)

As was the case for western flies, a higher proportion of eastern flies exhibited upwind flight to the eastern extract (63%) compared to the western extract (19%; $P=0.002$, 1 *df*), the apple blend (11%; $P<0.001$, 1 *df*), and the flowering dogwood blend (0%; $P<0.001$, 1 *df*). The difference between levels of upwind flight of eastern flies to their natal extract and the downy hawthorn blend was marginally significant (63 vs. 30%; $P=0.055$, 1 *df*). However, eastern mayhaw flies did not differ significantly in levels of upwind flight between their natal eastern extract vs. the hybrid *C. rufula* extract (63 vs. 70%, respectively; $P=0.610$, 1 *df*).

Behavioral Responses of Mayhaw Flies to Synthetic Mayhaw Blends Eastern and western mayhaw flies next were tested to synthetic blends of the complete set of GC-EAD active volatiles identified for western (designated Wc) and eastern mayhaw (designated Ec) (Table 2). The proportion of western flies displaying upwind flight to the 6-component Wc blend was 75% (Fig. 4a) and not significantly different from the 67% value exhibited by western flies to the *C. opaca* extract ($P=0.814$, 1 *df*). Similarly, the proportion of eastern flies displaying upwind flight to the 8-component Ec blend was 74% (Fig. 4b), a level equivalent to and not significantly different from the 63% value exhibited by eastern flies to the *C. aestivalis* extract ($P=0.419$, 1 *df*). These two results support the hypothesis that the Wc and Ec blends contain all of the key volatiles found in the extracts that elicit maximal response levels for western and eastern fly behavior, respectively. However, western and eastern flies displayed significantly reduced levels of upwind flight to their respective non-natal blends. The response level for western flies to the eastern Ec blend was 8% upwind flight (Fig. 4a), while the response level for eastern flies to the western Wc blend was 12% upwind flight (Fig. 4b).

We also tested eastern and western mayhaw-origin flies to a 9-component *C. rufula* hybrid blend (designated Hc) (Fig. 4a and b). The results were similar to those observed for the *C. rufula* adsorbent extract. Once again, western flies showed a low level of response to the Hc blend (8% upwind flight), while eastern flies responded in high proportion to the Hc blend (60% upwind flight).

Behavioral Responses of Mayhaw Flies to Modified Blends To better determine the impact of potential key volatile compounds responsible for the agonist and antagonist behavioral responses of mayhaw flies to natal and non-natal fruit volatiles, we tested three different modified blends with a focus on 3-methylbutan-1-ol and butyl acetate (Table 3). A total of 62 western mayhaw-origin flies (La Salle Parish, LA, USA, $N=27$; Grant Parish, LA, USA, $N=17$; Nacagdoches Co., TX, USA, $N=18$) were

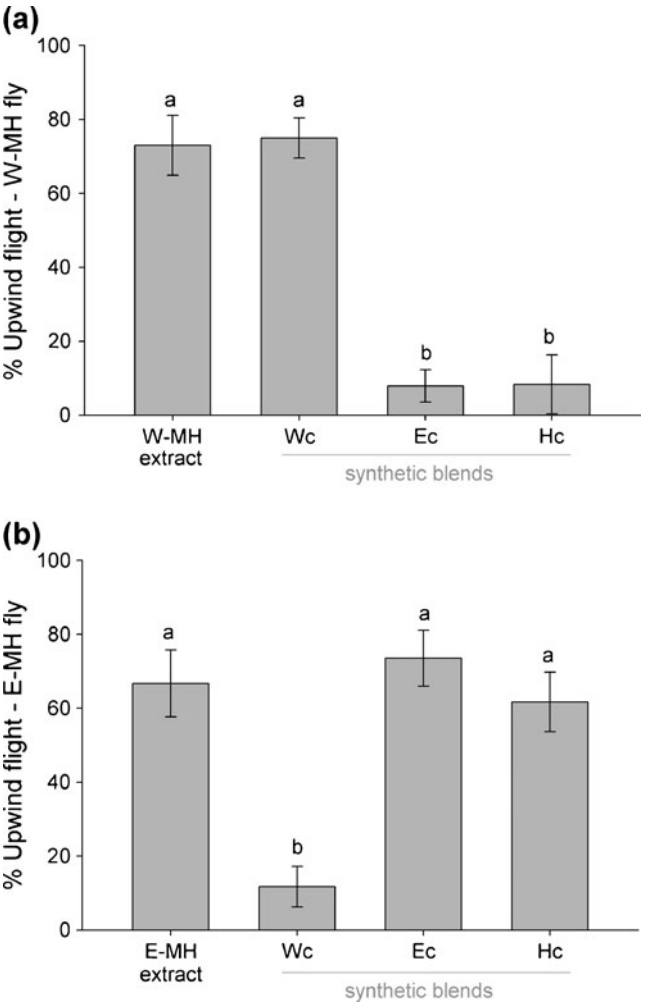


Fig. 4 Upwind flight responses (%±1 *SE*) of **a** western mayhaw-origin flies and **b** eastern mayhaw-origin flies to their natal whole fruit adsorbent extract and volatile blends of western (Wc), eastern (Ec), and "hybrid" mayhaw (Hc). W-MH and E-MH stand for western mayhaw and eastern mayhaw, respectively. Different letters on bars indicate significant differences ($P<0.05$)

tested to the modified mayhaw blends (Fig. 5). Western flies displayed reduced levels of upwind flight to the W1 (Wc plus butyl acetate) blend (10 vs. 75% upwind flight, respectively; $P<0.001$, 1 *df*), indicating that butyl acetate functions as an inter-specific behavioral antagonist on western fly behavior. Consistent with this hypothesis, western fly response to the eastern blend E1 (Ec minus butyl acetate) was not statistically different from that to the complete Wc blend (68 vs. 75%; $P=0.647$, 1 *df*). The critical importance of 3-methylbutan-1-ol was evidenced by the fact that no western flies exhibited upwind-directed flight to the modified E2 blend (E1 minus 3-methylbutan-1-ol). The tests with the modified blends also suggested that the compound pentyl hexanoate did not greatly affect flight behavior of western flies. Although pentyl hexanoate, like butyl acetate, is present in the eastern, but not western,

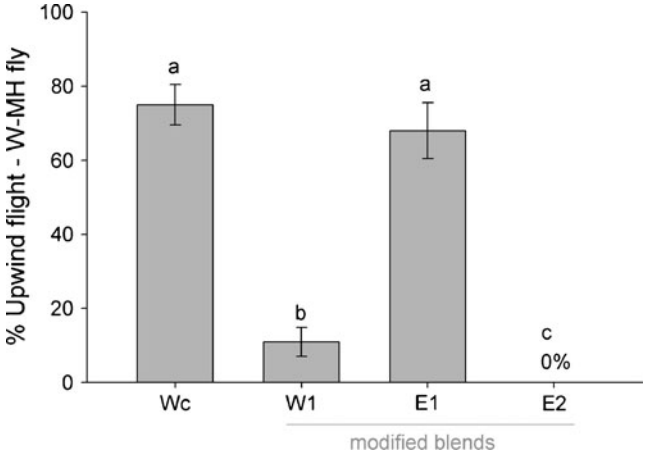


Fig. 5 Upwind flight responses (%±1 *SE*) of western mayhaw-origin flies to the complete western volatile blend (Wc), modified blend W1 (Wc plus butyl acetate), E1 (complete eastern mayhaw blend minus butyl acetate), and E2 (E1 minus 3-methylbutan-1-ol). W-MH stands for western mayhaw. Different letters on bars indicate significant differences ($P<0.05$)

blend (Table 3), western flies responded in high proportions (68%) to the modified eastern blend E1 that had butyl acetate removed, but still contained pentyl hexanoate.

A total of 34 eastern flies from Byron, GA, USA, were tested to the E1 and E2 blends (Fig. 6). Eastern flies exhibited a reduced level of upwind-directed flight to the E1 (Ec minus butyl acetate) blend (7% upwind flight) compared to the complete 8-component Ec blend (74% upwind flight; $P<0.001$, 1 *df*), suggesting that butyl acetate is a critical volatile for maximal response by eastern flies. Eastern flies showed no response (0% upwind-directed flights) to the E2 blend that lacked both butyl acetate and 3-methylbutan-1-ol.

Discussion

We developed synthetic volatile blends of western (*C. opaca*) and eastern (*C. aestivalis*) mayhaw fruit using GC-EAD and GC-MS analysis of SPME and whole fruit adsorbent collections. Western and eastern flies displayed high levels of upwind directed flight to their respective natal blends and a reduced response to almost all of the non-natal blends in flight tunnel assays, the lone exception being eastern mayhaw flies acceptance of the hybrid *C. rufula* blend. A key difference between the eastern and western blends is the presence of butyl acetate in the eastern blend. Addition or subtraction of butyl acetate to the synthetic blends demonstrated the critical importance of this volatile for stimulating upwind directional flight in eastern flies and for antagonizing (arresting) the upwind flight of western flies. This sharp contrast in preference behavior indicates that *R. pomonella* flies infesting western and eastern fruit differ in their host odor response phenotypes. The results for butyl acetate also can help to explain the behavioral responses of mayhaw flies to the fruit volatiles of the morphologically intermediate *C. rufula*, which may represent a hybrid between *C. opaca* and *C. aestivalis* in the state of Mississippi. We found that *C. rufula*, like the eastern mayhaw, *C. aestivalis*, contains butyl acetate in high levels. As a consequence, eastern flies tested against the *C. rufula* blend showed a similar level of upwind flight compared to their natal blend, while the upwind flight of western flies was antagonized by the hybrid blend (Fig. 4a and b). The apparent lack of *R. pomonella* infestation of *C. rufula* in Mississippi may thus be due to inadequate sampling of host fruit or some other abiotic or biotic factor such as dramatic year-to-year variability in the timing or amount of fruit set, and/or high interspecific competition, as the fruit of *C. rufula* would appear to present no olfactory barrier to colonization by eastern mayhaw flies.

A principal motivation for the current study was to investigate a potential role for southern mayhaw flies in the formation of the apple host race in the northern USA. In particular, it has been hypothesized that the introduction of a geographically differentiated, hawthorn-infesting form of *R. pomonella* from the South could be the source of the apple-infesting race in the North (Carson, 1989; although see Bush et al., 1989). This would imply that in the southern USA, a "pre-adapted" hawthorn-infesting population of *R. pomonella* exists that preferentially orients to apple fruit volatiles and is antagonized by downy hawthorn (*C. mollis*) fruit volatiles. However, in the current study, flies collected from both eastern and western fruit exhibited low response levels with the apple blend in flight tunnel assays (6 and 10% upwind flight, respectively). Indeed, the proportion of upwind flight exhibited by southern mayhaw

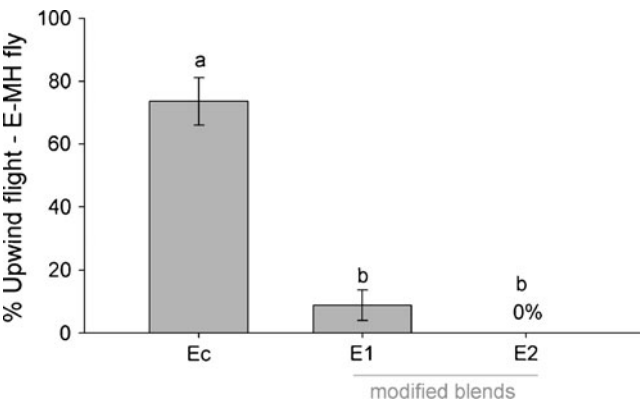


Fig. 6 Upwind flight responses (%±1 *SE*) of eastern mayhaw-origin flies to the complete eastern mayhaw blend (Ec), E1 (Ec minus butyl acetate), and E2 (E1 minus 3-methylbutan-1-ol). E-MH stands for eastern mayhaw. Different letters on bars indicate significant differences ($P<0.05$)

flies to the apple blend was lower than that shown for the northern downy hawthorn blend (Fig. 3). Further, all of these apple-responding flies also responded to their natal fruit extract or synthetic blend, indicating that they are examples of broadly responsive flies, ones that do not exhibit a high degree of response specificity for blend quality. Mayhaw infesting populations from the South, therefore, do not preferentially orient to apple and are not "pre-adapted", direct precursors to the apple race in the North. However, western and eastern mayhaw blends from the South do contain certain compounds present in the apple blend and absent in northern downy hawthorn blend (Table 2). In particular, both mayhaw blends and the apple blend contain butyl butanoate, propyl hexanoate, and hexyl butanoate, and eastern mayhaw and apple further share pentyl hexanoate. Mayhaw flies are thus capable of detecting certain components of the apple blend, and some southern flies are capable of responding to both hawthorn and apple blends, supporting the hypothesis that odor response phenotypes in the South may have contributed to pre-standing variation in northern hawthorn populations facilitating a rapid, sympatric shift to apple. Perhaps most importantly, northern downy hawthorn and both mayhaw blends share 3-methybutan-1-ol in common. 3-Methybutan-1-ol is not present in the apple blend (Table 2), and is an antagonist of upwind flight by apple-origin flies when added to the apple blend (Linn et al., 2005a). The lack of response of both eastern and western flies to modified blends lacking 3-methylbutan-1-ol, together with our previous findings for northern downy hawthorn flies (Linn et al., 2003; 2005a), supports the hypothesis that this compound functions as a critical volatile for hawthorn fly host discrimination.

Rather than a direct shift of southern mayhaw flies to apple to form the northern apple fly race, our current results instead provide evidence for the possible existence of differentiated host races (or even sibling species) of hawthorn-infesting *R. pomonella* in the South. Neither western nor eastern flies were attracted greatly to the northern downy hawthorn blend or to their respective non-natal mayhaw blend. These findings are consistent with differences in olfactory preference and discrimination of volatile mixtures contributing to prezygotic reproductive isolation between these populations if they were to come in geographic contact. In an accompanying study (Cha et al., 2011, this issue), we report fruit volatile and fly response differences for two additional hawthorn species in the South (the blueberry hawthorn, *C. brachyacantha*, and the green hawthorn, *C. viridis*), further supporting the hypothesis of southern hawthorn-infesting races of *R. pomonella*.

We now have developed active volatile blends for six different host fruit of *R. pomonella* group flies. Previously, we optimized volatile blends for apple (Zhang et al., 1999), northern downy hawthorn (Nojima et al., 2003a), and flowering dogwood (Nojima et al., 2003b). The addition of the three new western, eastern, and hybrid mayhaw blends in the current study will allow us to further characterize phenotypic patterns and elucidate the genetic architecture of host plant preference in the model *R. pomonella* system, thus leading to a greater understanding of the role that host volatile recognition plays in phytophagous insect speciation. Studies that compare the behavior of southern mayhaw flies to those of other southern *Crataegus* infesting populations will give us insight into the generality of host odor recognition as an isolating mechanism in the *R. pomonella* species complex and into the nature of fitness trade-offs posed by blends composed of overlapping components (Cha et al., 2011, this issue). Additionally, the importance of pre-standing variation in hawthorn flies to recognize certain specific fruit volatiles in apple may become clearer as we form a complete picture of host race formation among southern *R. pomonella* populations.

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However, the situation is different in the southern USA, where *R. pomonella* infests an array of several different hawthorn species that generally overlap in their geographic ranges, but differ in key traits such as fruiting time, host fruit color and size, and potentially fruit volatiles, thus presenting the opportunity for host-related divergence.

In a companion paper, we investigated the fruit volatile profiles for three related *Crataegus* species in the series *Aestivales*, collectively known under the common name of "mayhaw" in the South. Two of these species, the western mayhaw (*C. opaca*) and the eastern mayhaw (*C. aestivalis*), are infested by *R. pomonella*. A third described species, *C. rufula*, occurs primarily at the intersection of the eastern and western species' ranges between the lower Pearl River and Pascagoula River drainages in Mississippi and may represent a hybrid species; *C. rufula* does not appear to be infested by *R. pomonella* (Cha et al., 2011b, this issue). Our results are consistent with the possibility of endemic southern hawthorn-infesting host races of *R. pomonella* and do not support a "pre-adapted" apple race of the fly being present in the South (Cha et al., 2011b).

There are other hawthorn hosts of *R. pomonella* in the South, however, that could serve as a source for a pre-assembled apple race and/or shed additional insight into the evolutionary origins of apple volatile attraction. The fly also infests blueberry hawthorn, *C. brachyacantha* Englem. and Sarg. and green hawthorn, *C. viridis* L., as principal hosts. The blueberry hawthorn is so named for its characteristic dark blue, subglobose fruit. It is one of the tallest southern species in *Crataegus*, and is further distinguished by its small, waxy, crenulate-serrated leaves and small curved thorns (Sargent, 1933). Blueberry hawthorn fruit typically ripens from late August through September. Blueberry hawthorns are found in moist soil on the edge of forests bordering on low-lying prairies (Correll and Johnston, 1970). Its natural range is the "piney woods" region of eastern Texas through much of Louisiana, barely extending into adjacent counties in Arkansas and Mississippi.

The green hawthorn, *C. viridis*, has a broader range. It is found from southern Illinois southward to the coastal plain of Texas, eastward to the Florida panhandle and as far north as the Virginia piedmont. It is sparsely distributed through much of this range, only occurring in high densities west of the Mississippi River (Sargent, 1890). Green hawthorn is comprised of a complex of both sexual and apomictic individuals (Talent and Dickinson, 2005), and is distinguished from other hawthorns by dark green, acute, ovate leaves and small red-orange fruit (Vines, 1977). Green hawthorn has the latest fruiting phenology of southern hawthorns, with ripening not occurring until November. Unlike many hawthorn species, whose fruit abscises shortly after ripening, uninfested green hawthorn fruit often remains on the tree through winter. For this reason, a cultivar of green hawthorn known as "Winter King" has recently become popular with horticulturalists outside its native range.

Here, we expand on our previous studies and report on the identification of volatile blends for blueberry and green hawthorns through a combination of gas chromatography-electroantennographic detection (GC-EAD) and gas chromatography–mass spectrometric (GC-MS) analysis of solid phase microextraction (SPME) and adsorbent volatile collection samples from whole fruit. Behavioral flight tunnel assays also were conducted using the blueberry and green hawthorn blends and our previously developed apple, downy hawthorn, and flowering dogwood blends to test for possible olfactory pre-adaptation of flies that infest these two southern hawthorn species to apple. We report results showing again that both blueberry and green hawthorn-origin flies respond maximally to their natal fruit volatile blends and are relatively unresponsive to the alternative non-natal blends, consistent with endemic host race formation of *R. pomonella* on native southern hawthorns. The results also show that the volatile blend for green hawthorn flies, in particular, contains volatiles used by northern downy hawthorn and apple flies, and thus could be a potential source for genetic variation, thus contributing to olfactory fruit odor discrimination and host race formation in the North.

Methods and Materials

Details concerning methods and procedures for fruit volatile adsorbent and SPME sampling, GC-EAD and chemical analyses, synthetic blend development, and flight tunnel behavioral testing, can be found in the companion paper (Cha et al., 2011b, this issue). As done previously in Cha et al. (2011b), differences in the frequency of upwind flight in the tunnel were compared using Fisher's Exact Test in R (R development core team, Vienna, Austria).

Insects Flies used in the study were collected as larvae in infested host fruit from three different blueberry and seven different green hawthorn sites in the South (Fig. 1; Table 1) and reared to adulthood in the Notre Dame laboratory (Neilson and McAllan, 1965; Dambroski and Feder, 2006). Upon eclosion, adult flies were kept in a walk-in environmental chamber on an artificial diet (Fein et al., 1982). Adult flies at 0–7 and 10–21-d-old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively.

Fruit Blueberry hawthorn fruit was collected from Monroe, LA, USA, in 2007 (Fig. 1; Table 1). Green hawthorn fruit was collected from Dewey Wills Wildlife Management

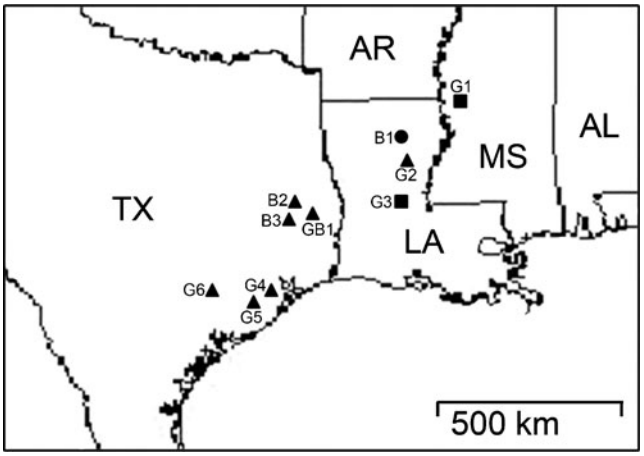


Fig. 1 Map of collection sites for blueberry hawthorn (B) and green hawthorn (G) fruit (*circles*), flies (*triangles*), or both (*squares*). Abbreviations for each of the southern States indicated are: AL, Alabama; AR, Arkansas; LA, Louisiana; MS, Mississippi; TX, Texas

Area, LA, in 2007 and Rolling Fork, MS, USA, in 2008 (Fig. 1; Table 1). Fruit collections were made off of trees in the field and shipped overnight to the Cornell lab for volatile characterization.

Chemicals Pentyl hexanoate, hexyl butanoate, butyl hexanoate, propyl hexanoate, pentyl butanoate, butyl butanoate, 1-octen-3-ol, 3-methylbutan-1-ol, 3-methylbutyl acetate, dimethyl trisulfide, β-caryophyllene (purities >98%), and dihydro-β-ionone (>90%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); butyl acetate (>99%) and pentyl acetate (>98%) from TCI America (Portland, OR, USA); ethyl acetate (99.9%) from Fischer Scientific (Pittsburgh, PA, USA). The compound (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was synthesized by oxidation of geraniol and then by Wittig reaction with methylenetriphenylphosphorane (Greenwald et al., 1963) and purified (>97% with >97% *E*-isomer by GC/MS) using flash chromatography on silica gel. The synthesis product was eluted with hexane and then subjected to Kugelrohr distillation.

Modified Synthetic Blends To determine the relative importance of individual volatiles in the blend, or those that might function as inter-specific behavioral antagonists for green and blueberry hawthorn-origin flies, we prepared six modified blends by adding and/or subtracting volatile compounds from the complete blends (Table 2). We concentrated on five different compounds: 3-methylbutan-1-ol, butyl acetate, propyl hexanoate, DMNT, and 1-octen-3-ol. Previous studies have shown that 3-methylbutan-1-ol is an essential volatile inducing agonistic behavior for northern downy hawthorn flies (Linn et al., 2005b) and also for southern mayhaw flies (Cha et al., 2011b, this issue). Butyl acetate was examined because it is present in the blueberry hawthorn, but not common in other hawthorn species found in blueberry hawthorn growing regions (e.g., western mayhaw, *C. opaca*). DMNT, 1-octen-3-ol, and propyl hexanoate are all compounds that are important for the northern apple, downy hawthorn, and dogwood host races. DMNT is unique to green hawthorn in the South and was previously shown to be essential for maximal upwind flight by northern downy hawthorn flies (Nojima et al., 2003a). The volatile 1-octen-3-ol is in both green and blueberry hawthorn, and is an essential volatile in the dogwood blend (Nojima et al., 2003b) but an antagonist to apple flies (Linn et al., 2005a). The modified blends were prepared so that each compound was always in the same amount as in corresponding complete blends, thus eliminating the potential effect of variation in concentration. For flight tunnel testing, we applied 200 μl of the modified blend to a rubber septum. The concentration of each modified blend is listed in Table 2.

Results

Identification of Volatiles from Green and Blueberry Hawthorn Fruit Green and blueberry hawthorn fruit were analyzed by using SPME and adsorbent extracts with GC-

Table 1 Collection sites for green hawthorn (*Crataegus viridis*) and blueberry hawthorn (*Crataegus brachyacantha*) fruit and flies as marked in Fig. 1

Map Name	Host plant species	Location	Latitude	Longitude
B1	*C. brachyacantha*	Co. Rd 841, Ouachita Parish, LA	32° 19′ N	90° 55′ W
B2	*C. brachyacantha*	SFA exp. forest, Nacogdoches Co, TX	31° 30′ N	94° 46′ W
B3	*C. brachyacantha*	Lufkin, Angelina Co, TX	31° 20′ N	94° 43′ W
GB1	Both	Marion Ferry Park, Angelina Co, TX	31° 30′ N	94° 32′ W
G1	*C. viridis*	Hwy 16, Rolling Fork, Sharkey Co, MS	32° 54′ N	90° 52′ W
G2	*C. viridis*	Fort Necessity, Caldwell Parish, LA	32° 02′ N	91° 49′ W
G3	*C. viridis*	Dewey Wills WMA, La Salle Parish, LA	30° 49′ N	90° 57′ W
G4	*C. viridis*	Brazos Bend SP, Fort Bend Co, TX	32° 28′ N	97° 45′ W
G5	*C. viridis*	Cedar Lanes, Wharton Co, TX	30° 25′ N	95° 21′ W
G6	*C. viridis*	Palmetto SP, Gonzalez Co, TX	28° 08′ N	96° 59′ W

Table 2 Relative ratio (%) of volatile compounds in the complete green (Gc) and blueberry (Bc) synthetic blends and their modified blends (G1, G2, B1, B2, B3 and B4). We prepared the modified blends so that the same compound was always in the same amount as in corresponding complete blends, thus eliminating the potential effect of variation in concentration. For flight tunnel tests, 200 μl of Gc or Bc (prepared at 1.0 μg/μl), thus 200 μg of total volatile compounds, were loaded to a rubber septum. Ratio and concentration of modified blends (G1, G2, B1, B2, B3 and B4) were formulated as found in Gc or Bc. For flight tunnel tests of the modified blends, we also loaded 200 μl of G1, G2, B1, B2, B3 or B4 to a rubber septum. Bold characters indicate the compounds modified. DMNT indicates (3*E*)-4,8-dimethyl-1,3,7-nonatriene

Chemicals	Synthetic blends (%)							
	Gc	G1	G2	Bc	B1	B2	B3	B4
3-methylbutan-1-ol	5	5		0.6	0.6	0.6	0.6	0.6
Butyl acetate				50	50			50
Pentyl acetate				3.5	3.5	3.5	3.5	3.5
Butyl butanoate	19.5	19.5	19.5	9	9	9	9	9
Propyl hexanoate	1.5		1.5					
Butyl hexanoate	24	24	24	16.8	16.8	16.8	16.8	16.8
Hexyl butanoate	24	24	24	16.8	16.8	16.8	16.8	16.8
Pentyl hexanoate	2.5	2.5	2.5					
Pentyl butanoate	2.5	2.5	2.5	3	3	3	3	3
DMNT	20.5	20.5	20.5				20.5	20.5
1-octen-3-ol	0.5		0.5	0.3				
Total %	100	98	95	100	99.7	49.7	70.2	120.2
Concentration (μg/μl)	1.0	0.98	0.95	1.0	0.997	0.497	0.702	1.202

EAD and GC-MS. Figure 2a and b shows GC-EAD recordings from the antennae of a representative green or blueberry hawthorn-origin fly exposed to an extract sample from ripe fruit. We note that whereas the trace indicates the presence of some unidentified peaks with EAD responses, these responses were not present in the majority (>75%) of GC-EAD runs examined. The relative ratio of the EAD active compounds estimated by GC-MS for the whole green and blueberry hawthorn fruit adsorbent extracts is given in Table 3.

For green hawthorn (*C. viridis*), fruit collected from two sites (Dewey Wills, LA, USA 2008; Sharkey Co., MS, USA 2008) were analyzed. A total of 24 different antennal pairs of 21 female and three male green hawthorn-origin flies (1–10 replicate runs/pair) was tested. The compounds that consistently elicited EAD responses were identified as (Ia) 3-methylbutan-1-ol, (IIa) 1-octen-3-ol, (IIIa) butyl butanoate, (IVa) pentyl butanoate, (Va) propyl hexanoate, (VIa) (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (VIIa) butyl hexanoate, (VIIIa) hexyl butanoate, and (IXa) pentyl hexanoate (Table 3).

For Blueberry hawthorn (*C. brachyacantha*), fruit collected at Ouachita Parish, LA, USA, in 2007 was analyzed. A total of seven different antennal pairs of 5 female and 2 male blueberry hawthorn-origin flies (1–6 replicate runs/pair) was tested using SPME and the extract in the GC-EAD analysis of *C. brachyacantha* volatiles. The corresponding compounds eliciting consistent EAD activity were identified as (Ib) 3-methylbutan-1-ol, (IIb) butyl acetate, (IIIb) 1-octen-3-ol, (IVb) butyl butanoate, (Vb) pentyl butanoate, (VIb) butyl hexanoate, (VIIb) hexyl butanoate, and (VIIIb) pentyl hexanoate (Table 3).

Behavioral Responses of Green and Blueberry Hawthorn-Origin Flies to Natal Adsorbent Extracts and Non-Natal Northern Volatile Blends A total of 26 green hawthorn-origin flies from Fort Necessity, LA, USA (N=5), Brazos Bend, TX, USA (N=4), and Rolling Fork, MS, USA (N=17) and 17 blueberry hawthorn flies from the Stephen Austin Experimental Forest, near Nacogdoches, TX, USA were tested in the flight tunnel to adsorbent extracts from green and blueberry hawthorn, and to 200 μg of the previously developed apple, downy hawthorn, and flowering dogwood blends (Fig. 3). For green hawthorn-origin flies, the response patterns were not statistically different among the three sites for any of the host volatiles tested, and so we present the combined results. A higher proportion of green hawthorn-origin flies displayed upwind directed flight to the green hawthorn extract (69%) than to the blueberry hawthorn extract (34%; P=0.002, 1 *df*), the apple blend (4%; P=0.005, 1 *df*), and the downy hawthorn blend (23%; P=0.002, 1 *df*) (Fig. 3a). No green hawthorn-origin fly responded to the dogwood blend. Similarly, a higher proportion of blueberry hawthorn-origin flies displayed upwind directed flight to the blueberry hawthorn extract (76%) than the green hawthorn extract (24%; P=0.0121, 1 *df*), the downy hawthorn blend

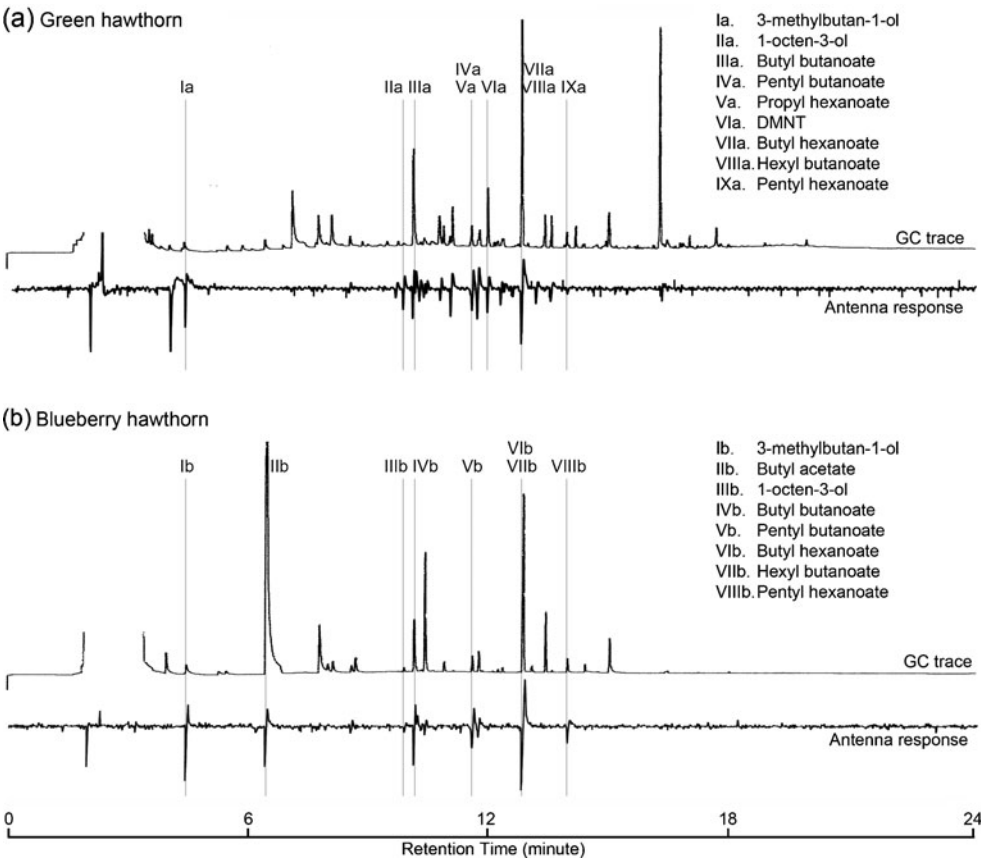


Fig. 2 Simultaneous recorded GC-EAD responses of adult green and blueberry hawthorn fly antennae to adsorbent samples of green and blueberry hawthorn, respectively. Different traces indicate antennal response of (**a**) green hawthorn-origin flies to green hawthorn fruit adsorbent samples and (**b**) blueberry hawthorn-origin flies to blueberry hawthorn fruit adsorbent samples. DMNT indicates (3*E*)-4,8-dimethyl-1,3,7-nonatriene

Table 3 The relative ratios (%) of volatile compounds used in different synthetic blends. Ratios of the green and blueberry hawthorn blends were based on GC/MS analyses of volatile adsorbent samples collected from green and blueberry hawthorn fruits, respectively. The ratio of apple, red hawthorn and dogwood blends were previously developed by Zhang et al. (1999) and Nojima et al. (2003a,b). DMNT indicates (3*E*)-4,8-dimethyl-1,3,7-nonatriene.

Chemicals	Synthetic blends (%)				
	Green hawthorn (Gc)	Blueberry hawthorn (Bc)	Apple	Downy hawthorn	Flowering dogwood
3-methylbutan-1-ol	5	0.6		4	27.5
Butyl acetate		50			
Pentyl acetate		3.5			
Butyl butanoate	19.5	9	10		
Propyl hexanoate	1.5		4		
Butyl hexanoate	24	16.8	37	0.01	
Hexyl butanoate	24	16.8	44		
Pentyl hexanoate	2.5		5		
3-methylbutyl acetate				1.5	0.9
Pentyl butanoate	2.5	3			
Ethyl acetate				94.32	54.9
DMNT	20.5			0.07	
Dihydro-β-ionone				0.1	
Dimethyl trisulfide					1.9
1-octen-3-ol	0.5	0.3			9.1
β-caryophyllene					5.8

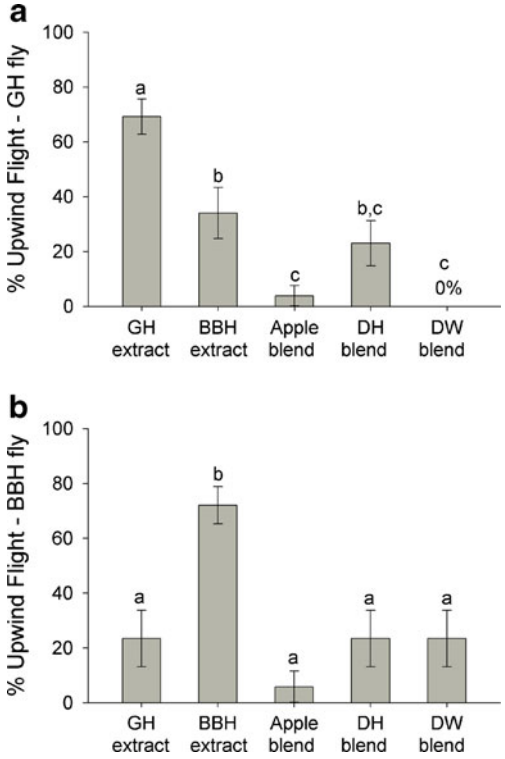


Fig. 3 Upwind flight responses (% ± 1 *SE*) of (**a**) green hawthorn-origin flies and (**b**) blueberry hawthorn-origin flies to green and blueberry hawthorn fruit adsorbent extracts as well as previously described synthetic volatile blends for three northern USA host plants: apple, downy hawthorn, and flowering dogwood. List of abbreviations used and what they stand for: GH, green hawthorn; BBH, blueberry hawthorn; DH, downy hawthorn; DW, flowering dogwood. Different letters on bars indicate significant differences ($P<0.05$)

(24%; $P=0.0121$, 1 *df*), and the flowering dogwood blend (24%; $P=0.0121$, 1 *df*). Only one of the 17 blueberry hawthorn-origin flies (6%) responded to the apple blend (Fig. 3b).

Behavioral Response of Green and Blueberry Hawthorn-Origin Flies to the Synthetic Blends A total of 26 green hawthorn-origin flies from four different sites (Fort Necessity, LA, USA [$N=8$]; Rolling Fork, MS, USA [$N=13$]; Brazos Bend, TX, USA [$N=1$]; and Cedar Lane, TX, USA [$N=4$]) were tested to both the green hawthorn extract and the 9-component synthetic blend developed for green hawthorn fruit (designated Gc). A total of 26 blueberry hawthorn-origin flies from two different sites (Nacogdoches, TX, USA [$N=19$]; and Marion Ferry, TX, USA [$N=7$]) were tested to the blueberry hawthorn extract and to an 8-component synthetic blend of the complete set of EAD active volatiles identified from fruit (designated Bc). For both fly host populations, there was no difference in the level of upwind directed flight with the Gc or Bc blend compared to the respective extracts (for green hawthorn 81 vs. 69%, respectively, $P=0.523$, 1 df; for blueberry 88 vs. 65%, respectively, $P=0.098$, 1 *df*). These results support the hypothesis that the Gc and Bc blends contain all of the key volatiles required for maximal levels of green and blueberry hawthorn upwind flight behavior. We, therefore, used the Gc and Bc blends as a baseline for comparison in subsequent tests to investigate the behavioral responses of flies to specific volatile compounds (see section below on Behavioral Responses of Flies to Modified Blends).

Behavioral Responses of Flies to Modified Blends Analysis of the flight responses of green and blueberry hawthorn-origin flies to two complete synthetic (Gc and Bc) and six modified blends (G1, G2, B1, B2, B3, and B4; Table 2) provided evidence for DMNT as a behavioral agonist for green hawthorn flies and a behavioral antagonist for blueberry hawthorn flies, and butyl acetate as a behavioral agonist to blueberry hawthorn flies and a behavioral antagonist for green hawthorn flies (Figs. 4 and 5). There also was evidence for 3-methylbutan-1-ol as an important volatile for maximal upwind flight levels by both green and blueberry hawthorn flies, while 1-octen-3-ol (a critical volatile for the dogwood flies; Linn et al., 2005b) and propyl hexanoate (found in the apple blend) had little influence on fly behavior.

For the 85 green hawthorn flies from Dewey Wills, LA, USA ($N=20$); Fort Necessity, LA, USA ($N=26$); Rolling Fork, MS, USA ($N=28$); Brazos Bend, TX, USA ($N=4$); and Cedar Lane, TX, USA ($N=7$), there was no significant difference in the level of upwind directed flight between the complete Gc blend and the G1 blend (Gc minus 1-octen-3-ol and propyl hexanoate) (73 vs. 78%; $P=0.594$, 1 *df*), suggesting that 1-octen-3-ol and propyl hexanoate were not essential for maximal upwind flight responses by green hawthorn flies (Fig. 4a). Of the 85 flies, 40 (Dewey Wills, LA, USA [$N=20$]; Fort Necessity, LA, USA [$N=11$]; Rolling Fork, MS, USA [$N=6$]; and Brazos Bend, TX, USA [$N=3$]) also were tested against blend G2 (Gc minus 3-methylbutan-1-ol), with upwind directed flight responses significantly lower than with Gc (10 vs. 78%; $P<0.001$, 1 *df*). In addition, the same 26 green hawthorn flies tested above to both the green hawthorn adsorbent extract and Gc were tested to the complete blueberry hawthorn blend (Bc) and modified B1 (Bc minus 1-octen-3-ol). Green hawthorn flies exhibited low response levels to both Bc and B1 (23 and 4% upwind flight behavior; Fig. 4b).

To test the effect of butyl acetate and DMNT on green hawthorn-origin flies, we performed the following comparisons. The 85 green hawthorn flies tested above to Gc and G1 also were tested to modified blueberry hawthorn blend B2 (B1 minus butyl acetate), to which they showed an increased level of upwind directed flight compared with Bc (47 vs. 24%; $P=0.04$; Fig. 4b), suggesting that butyl

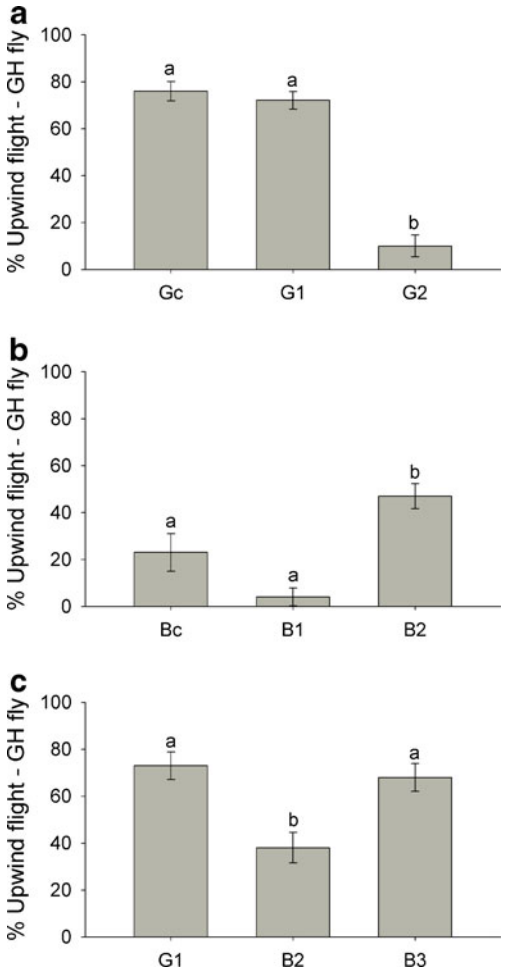


Fig. 4 Upwind flight responses (% ± 1 *SE*) of green hawthorn-origin flies to (**a**) Gc: the complete green hawthorn synthetic blend, G1: a modified synthetic green hawthorn blend missing 1-octen-3-ol and propyl hexanoate, and G2: a modified version of Gc missing 3-methylbutan-1-ol, (**b**) Bc: the complete blueberry hawthorn synthetic blend, B1: a modified blueberry hawthorn blend missing 1-octen-3-ol, and B2: a version of B1 missing butyl acetate, and (**c**) G1, B2, and B3: a modified version of B2 containing DMNT. GH indicates green hawthorn. Different letters on bars indicate significant differences ($P<0.05$)

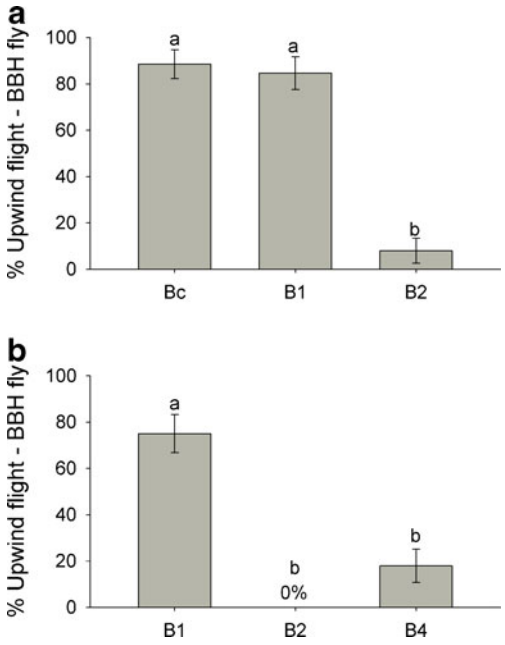


Fig. 5 Upwind flight responses (% ± 1 *SE*) of blueberry hawthorn-origin flies to (**a**) Bc: the complete blueberry hawthorn synthetic blend, B1: a modified version of Bc missing 1-octen-3-ol, and B2: a modified version of B1 missing butyl acetate, and (**b**) B1, B2, and B4: a modified version of B1 containing DMNT. BBH indicates blueberry hawthorn. Different letters on bars indicate significant differences ($P<0.05$)

acetate, a unique compound to blueberry hawthorn fruit among the southern hawthorn species, functions as an inter-specific behavioral antagonist on the flight behavior of green hawthorn flies. A total of 56 additional green hawthorn flies from Brazos Bend, TX, USA (N=31), Dewey Wills, LA, USA (N=18), Marion Ferry, TX, USA (N=5), and Palmetto State Park, TX, USA (N=2), were tested to the modified blueberry blend B3, as well as to B2 and G1 (Fig. 3c). Modified blend B3 represented blend B2 with the compound DMNT added, making B3 very similar in composition (except for pentyl acetate in B3) to the modified G1 blend. For these 56 green hawthorn flies, the upwind directed flight response to B3 (68%) was higher compared to B2 (38%; $P<0.001$, 1 *df*) and was similar to that for G1 (73%; $P=0.66$), suggesting that DMNT is a critical volatile in the blend that green hawthorn flies use to locate green hawthorn trees (Fig. 4c).

To test the effect of 1-octen-3-ol, butyl acetate, and DMNT on blueberry hawthorn flies, we performed the following comparisons. For the 26 blueberry hawthorn-origin flies tested above from Nacogdoches, TX, USA (N=19), and Marion Ferry, TX, USA (N=7), there was no significant difference in the level of upwind directed flight between the complete Bc blend and the modified B1 blend (Bc minus 1-octen-3-ol) (88 and 85% upwind flight, respectively), suggesting that 1-octen-3-ol does not greatly affect upwind flight by blueberry hawthorn flies (Fig. 5a). However when butyl acetate was removed from the B1 blend to create blend B2, fewer blueberry hawthorn flies exhibited upwind directed flights (8%) compared with B1 (88%; $P<0.001$, 1 *df*), supporting the essential role of butyl acetate for blueberry hawthorn flies (Fig. 5a). Further support for a major role of butyl acetate came from a test of 28 blueberry flies collected from Lufkin, TX, USA in 2009. These flies also displayed a dramatic decrease in response when butyl acetate was not present in the blueberry hawthorn blend (75% upwind flight to B1 vs. 0% to B2; $P<0.001$, 1 *df*) (Fig. 5b). Moreover, addition of DMNT to B1 to create modified blend B4 also reduced upwind directed flight behavior for the 28 Lufkin, TX, USA, flies (75% upwind

flight to B1 vs. 18% to B4; $P<0.001$, 1 *df*), suggesting that blueberry hawthorn-origin fly flight behavior was antagonized by the presence of DMNT, a key green hawthorn volatile (Fig. 5b) in the mixture.

Discussion

We developed synthetic volatile blends for green (*C. viridis*) and blueberry hawthorn (*C. brachyacantha*) fruit using GC-EAD and GC-MS analysis of SPME and adsorbent samples. In the flight tunnel, green and blueberry hawthorn-origin flies displayed maximal levels of upwind directed flight to their respective natal blends and reduced response levels to their non-natal blends. The reduced response levels were due to differences in key volatiles that comprised the behaviorally active blend for each host, and to the presence of non-natal fruit volatiles that function as behavioral antagonists.

Two key differences were discovered between the green and blueberry hawthorn blends. The first was the presence of DMNT in the green hawthorn blend and its absence from the blueberry hawthorn blend. Addition and subtraction of DMNT to synthetic green and blueberry hawthorn blends demonstrated the critical importance of the compound for stimulating upwind oriented flight for green hawthorn-origin flies and for antagonizing the upwind flight of blueberry hawthorn flies. The compound DMNT has not been found in the fruit of other southern hawthorn species besides green hawthorn (blueberry hawthorn and three mayhaw species; Cha et al., 2011b, this issue), suggesting that it may be a unique volatile in the blend distinguishing *C. viridis*. The second difference is the presence of butyl acetate in the blueberry hawthorn blend and its absence from the green hawthorn blend. Addition and subtraction of butyl acetate to the respective synthetic blends showed the critical importance of this volatile for stimulating upwind oriented flight in blueberry flies and for antagonizing the upwind flight of green hawthorn flies. The striking contrast in preference behavior indicates that southern *R. pomonella* flies that infest green vs. blueberry hawthorn differ in their host odor response phenotypes. Butyl acetate has not been found in the fruit of other southern hawthorn species that co-occur with the blueberry hawthorn across its range in eastern Texas and Louisiana (e.g., western mayhaw, *C. opaca*), although the compound is present in the more eastern distributed mayhaws, *C. aestivalis* and *C. rufula* (Cha et al., 2011b, this issue). Butyl acetate may, therefore, be a key compound in the blend that distinguishes *C. brachyacantha* throughout its geographic distribution in the South. The results for DMNT and butyl acetate continue a pattern we first observed in northern *R. pomonella* populations (Linn et al., 2005a, b) involving positive orientation of flies to key mixtures of volatile components present in the adsorbent extract profiles of their natal host fruit, and antagonism or arrestment of upwind flight when unique compounds found in alternative non-natal fruit are added to the natal blend. Antagonism is important because it indicates that when flies shift to a new host plant, not only do they evolve to positively behaviorally respond to the volatile profile of the new fruit (where offspring survivorship is presumably higher) but also to avoid certain volatiles of other, alternative host fruit (where fitness should be lower).

The green hawthorn and blueberry hawthorn synthetic blends also showed similarities. Perhaps noteworthy, both blends, as well as the northern downy hawthorn, the dogwood blend, and the three southern mayhaw blends, share 3-methybutan-1-ol in common. Moreover, 3-methybutan-1-ol is not present in the apple blend, and is a potent behavioral antagonist for apple-origin fly flight behavior (Linn et al., 2005a). Our current results, therefore, further underscore that 3-methybutan-1-ol may be an essential component of behaviorally active volatile blends for hawthorn infesting *R. pomonella* flies. In comparison, the volatile 1-octen-3-ol that we previously showed to be a key agonist volatile for dogwood fly behavior and a behavioral antagonist for apple flies (Linn et al., 2005a), while present in both the green hawthorn and blueberry hawthorn blend, did not significantly affect the upwind flight of green or blueberry hawthorn flies.

Because of limitations in the number of available flies, we were not able to conduct exhaustive subtraction assays for all of the compounds in the respective natal blends. Thus, it is not possible to definitively say whether any of the remaining compounds in the green hawthorn and blueberry hawthorn synthetic blends (e.g., butyl hexanoate and hexyl butanoate) are essential for behavioral activity and discrimination. We also recognize that important differences in the ratio of common compounds could be important (e.g., Cha et al., 2011a). For example, butyl hexanoate is an important volatile for the northern downy hawthorn (*C. mollis*) flies, but is present in very low quantities compared to the apple blend (<1 vs. 44%; Nojima et al., 2003a), and high proportions of butyl hexanoate in the northern downy hawthorn blend have been shown to act antagonistically on upwind flight responses (Linn et al., 2005a).

Coupled with our results for three mayhaw host plants in the southern USA (Cha et al., 2011b, this issue), the current study does not support the hypothesis that the introduction of a geographically differentiated, southern hawthorn-infesting form of *R. pomonella* was the direct source of the apple-infesting race of the fly in the North (Carson,

1989; see Bush et al., 1989). In the current study, flies collected from both green and blueberry hawthorn fruit exhibited low response levels with the apple blend in flight tunnel assays (0 and 6% upwind directed flight, respectively). Indeed, the proportion of upwind directed flight exhibited by southern green hawthorn and blueberry hawthorn-origin flies to the apple blend was lower than that shown for the downy hawthorn blend (27 and 25%, respectively). Green and blueberry hawthorn-infesting populations of *R. pomonella* therefore do not preferentially orient to apple and are not "pre-adapted", direct precursors to the apple race in the North. However, just like southern mayhaws (Cha et al., 2011b, this issue), both green and blueberry hawthorn fruit from the South also contain certain compounds present in the apple blend and absent in the fruit of the downy hawthorn, the major hawthorn host attacked by *R. pomonella* in the North. In particular, mayhaw, green hawthorn, and the apple blend all contain butyl butanoate, propyl hexanoate, butyl hexanoate, hexyl butanoate, and pentyl hexanoate. Blueberry and green hawthorn flies are thus capable of detecting certain components of the apple blend, and some southern flies are capable of responding to both hawthorn and apple blends, supporting the hypothesis that odor response phenotypes in the South may have contributed to pre-standing variation in northern hawthorn populations facilitating a rapid, sympatric shift to apple. Rather than a pre-adapted apple race in the South, our results instead provide more evidence for the possible existence of differentiated races (or even sibling species) of hawthorn-infesting *R. pomonella* in the South.

We now have developed active synthetic blends for eight different host fruit of *R. pomonella* group flies. With the addition of the green hawthorn and blueberry hawthorn blends in the current study, we have now characterized fruit volatile profiles for most of the major hawthorn hosts infested by *R. pomonella* across the northern and southern USA. Development of these blends will allow us to further characterize phenotypic patterns and elucidate the genetic architecture of host plant preference in the classic *R. pomonella* system, leading to a greater understanding of the role that host volatile recognition plays in phytophagous insect speciation (Smadja and Butlin, 2009). The importance of pre-standing variation in hawthorn flies to recognize certain specific fruit volatiles in apple may now become clearer as we approach a complete picture of host race formation among southern *R. pomonella* populations.

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converted by glucosidases to the more reactive aglycones when plant cells are damaged by insect herbivory and pathogen infection (Niemeyer 2009).

While largely viewed as constitutive defenses, individual benzoxazinoid pools can be influenced greatly by biotic stresses. For example, insect herbivory can increase DIMBOA levels in wounded leaves and shoots of maize (Gutierrez et al. 1988; Erb et al. 2009). In other studies, DIMBOA-Glc [2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose] levels decreased with insect and pathogen infection, but there was a corresponding increase in the concentration of HDMBOA-Glc [2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose] (Oikawa et al. 2004). Increases in HDMBOA-Glc result from the methylation of existing pools of DIMBOA-Glc by a DIMBOA-Glc 4-O-methyltransferase (Oikawa et al. 2001, 2002), but there also is evidence for *de novo* biosynthesis of HDMBOA-Glc in response to endogenous peptide defense signals (Huffaker et al. 2011). Similar to DIMBOA, HDMBOA has been shown to have a negative impact on insect growth and development. When incorporated into artificial diet, HDMBOA-Glc effectively decreased the survival and fecundity of the aphid, *Metopolophium dirhodum* (Walker) at concentrations as low as 1 mM (Cambier et al. 2001). In a nitrogen restricted diet, HDMBOA-Glc completely inhibited the growth of southwestern corn borers (*Diatraea grandiosella* Dyar) (Hedin et al. 1993). Although there is evidence that benzoxazinoids function in insect defense, little is known regarding their function with respect to ECB stem herbivory.

In maize leaves, the plant defense hormone, jasmonic acid (JA) previously has been shown to regulate the concentration and composition of benzoxazinoids (Oikawa et al. 2001). Treating leaves exogenously with JA resulted in increased HDMBOA-Glc and decreased DIMBOA-Glc levels similar to those observed with biotic stresses. JA is a long established endogenous regulator of induced plant defenses, including proteins that can have anti-nutritive or toxic effects on insects (Howe and Jander 2008).

Plant defense signaling often involves cross talk among multiple endogenous phytohormones that provides resistance against invading pathogens and insects while reducing fitness costs to the plant (Koornneef and Pieterse 2008). JA frequently works synergistically with the phytohormone ethylene (ET) in regulating defense responses in plants. For example, kauralexins, a class of maize diterpenoids, are induced in young maize stems in response to the combination of JA and the ethylene releasing compound, ethephon, but not JA alone (Schmelz et al. 2011). Kauralexins also increased in response to ECB stem herbivory. In a choice assay, ECB avoided stem tissues treated with kauralexins, thus indicating that these compounds have anti-feedant activity (Schmelz et al. 2011). Contrary to ET, phytohormones such as salicylic acid (SA) and indole-3-acetic acid (IAA) generally negatively regulate JA signaling (Koornneef and Pieterse 2008).

Although herbivore induced defenses in leaves have been studied widely in crop plants, much less is known concerning insect induced defenses in stems. In the current study, we characterized chemical changes that occur in maize stem internode tissues in response to ECB herbivory. We measured changes in concentrations of the defensive compounds, benzoxazinoids and kauralexins. We also measured changes in phytohormones in an effort to understand how these signals may influence the expression of maize defenses. Additionally, we determined the impact of ECB-induced maize defenses on the growth of ECB.

Methods and Materials

Plant and Insect Materials. Zea mays var. Golden Queen seeds were germinated in three gallon pots, 4 plants per pot, in MetroMix 200 (Piedmont Pacific, Statham, GA, USA) containing 1.9 gl^{-1} 14-14-14 Osmocote (Scotts, Marysville, OH, USA). Plants were maintained in a greenhouse supplemented with 400 W high pressure sodium lamps (Higuchi Inc., Plano, TX, USA) and multi-vapor lamps (GE, Cleveland, OH, USA) for a 12-h photoperiod. Relative humidity was 70%, and temperature was maintained at 24°C: 28°C (night: day). Plants were watered daily and fertilized weekly with 1.25 mg L^{-1} Peters 20-20-20 water soluble fertilizer (Scotts). Plants used for studies were approximately 30 d-old and had 11–13 leaves. ECB were supplied by Benzon Research (Carlisle, PA, USA). Prior to infesting plants with ECB, newly molted fifth instar larvae were fed maize whorl tissue overnight.

Experimental Treatments For controlled establishment of ECB herbivory, a hole was made with a #1 cork borer in the internode tissue in the middle of the 2nd and 3 rd nodes from the base of the stem. ECB were introduced by using a modified 200 μl pipette tip; 2 cm of the narrow end of the tip was removed to allow the ECB to move into the stem, which promoted feeding at this site. At indicated time points, approximately 2 cm stem sections were collected around the initial site of wounding. As a damage control, plants were wounded with a cork borer as described above, and samples were collected around the wound site during the same sample periods. Corresponding tissue samples also were collected from plants that were not previously damaged. For time course studies, three biological replicates were collected, and each replicate was comprised of tissue pooled from 3 different plants.

For the no choice feeding assay, fifth instars were introduced above the second node as described above. An

equal number of plants were wounded using the cork borer or were left undamaged. After 24 h, 2 cm stem sections surrounding the initial damage site were collected for both the ECB and wound treatments. Developmentally similar tissue was collected from the non-treated control plants. To estimate concentrations of benzoxazinoids and kauralexins present at 24 h, samples were collected and analyzed from 6 representative plants for each treatment.

To examine the effects of exogenously applied JA and ET precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), a hole was made with a #1 cork borer in the middle of the 2nd and 3rd nodes from the base of the stem and a 60 µl solution of JA–Na^+, [methyl jasmonate (Bedoukian Research, Danbury, CT, USA) was saponified with 10 M NaOH, diluted with excess H_20, passed through C18 solid phase extraction column, washed with H_20, eluted with 4:1 MeOH:H_20 and evaporated to yield JA–Na+], ACC (Sigma-Aldrich Corp., St. Louis, MO, USA), or a combination of JA–Na^+ and ACC prepared in water was applied to the wound site. Total applied amounts were 660 nmol $plant^{-1}$ JA–Na^+ and 300 nmol $plant^{-1}$ ACC. The wound site was sealed with parafilm to prevent drying and samples (N=5) were collected after 24 h.

Biochemical Analysis of Defense Metabolites and Phytohormones Concentrations of the defense metabolites, anthranilate, indole, and kauralexins, and the phytohormones, JA, SA, and IAA were measured using the vapor phase extraction method described by Schmelz et al. (2004). Concentrations of kauralexins A1–A3 and B1–B3 (Schmelz et al. 2011) were combined and reported as total kauralexins. Concentrations of total JA were calculated as a sum of the concentrations of *trans*- and *cis*-JA isomers. Benzoxazinoids were measured using HPLC as previously described (Huffaker et al. 2011). Ethylene emission was measured by sampling 1 ml of headspace from maize stem sections sealed in 27 ml plastic tubes followed by GC analysis (Schmelz et al. 2003a).

Gene Expression Analysis RNA from approximately 200 mg of stem tissue was extracted using a modified TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) protocol. To help remove contaminants, the chloroform phase separation step was completed two times. RNA was precipitated using 350 µl isopropanol, and samples were washed twice with 75% (v/v) ethanol. Two µg of RNA were reverse transcribed into cDNA using RETROscript® First Strand Synthesis Kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time PCRs consisted of 1x Power SYBR Green Master mix (Applied Biosystems), 300 nM each of forward and reverse primers, 0.8 µl of cDNA template and nuclease-free ddH_2O to a final volume of 20 µl. Amplification was performed using the ABI 7300 sequence detection system (Applied Biosystems) following standard thermal profile conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data were analyzed with SDS 1.3.1 software (Applied Biosystems). Relative expression levels were determined for three independent biological replicates that consisted of a pool from 3 plants, and all the reactions were run in triplicate. Threshold cycle (Ct) values for *anthranilate synthase subunit 2* (*ASsub2*), *anther ear 2* (*An2*), and *allene oxide synthase* (*AOS*) were normalized to *elongation factor 1α* (*EF 1α*) and the values for *1-aminocyclopropane-1-carboxylate oxidase* (*ACC Ox*) was normalized to the housekeeping gene encoding *ribosomal protein 17* (*RPL17*). As per Livak and Schmittgen (2001), ΔC_T *vs*. log input amount of RNA were plotted to determine which primer pairs had equivalent efficiencies (Supplemental Fig. 1). The levels of each gene transcript were calculated relative to its corresponding untreated control. Fold-changes of RNA transcripts were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The specificities of real-time PCR products were verified on 1% agarose gels. The gene-specific oligonucleotides that were designed to span an exon-exon junction to avoid amplication of genomic DNA are listed in Table 1.

No Choice Feeding Assay with ECB As described above, stem sections (2 cm) were collected from control, wound, and ECB damaged tissues, weighed, and placed into sterile 12-well tissue culture plates (Fisher Scientific, Vernon Hills, IL, USA). For each stem section, a newly molted 5th instar ECB that had been previously maintained on maize leaf tissue was weighed and then placed in the well with the stem tissue. Newly molted final instars were used because typically older larvae tunnel into maize stems, whereas younger larvae feed on leaves. After 24 h, both the stem tissue and ECB larvae were weighed to determine the amount of tissue consumed and the percent weight gain for each ECB. A total of 13 ECB were used for each treatment.

Statistical Analyses With the exception of the percent weight gain data, all data were square-root transformed prior to statistical analysis (Zar 1999). The percent weight gain data were arcsine square root transformed (Zar 1999). Analysis of variance (ANOVAs) and Tukey HSD tests were used to make multiple comparisons among control, wound, and ECB samples. All statistical analyses were performed with JMP 4.0 (SAS Institute, Cary, NC , USA).

Results

ECB Stem Herbivory Induces Accumulation of Maize Chemical Defenses. Transcript levels of *ASsub2*, a gene predicted to encode an anthranilate synthase (Huffaker et al.

Table 1 Primer sequences used to amplify biosynthetic enzymes

Symbol	NCBI Accession No.	Forward primer	Reverse primer	Amplicon Size (bp)
ASsub2	NM_001159139	ATCATTGCAGACTGTTCTTGA	ATGAGTCCATCTTCAGTCCAT	288
An2	AY562490	TGTTCTTGTGAAGGCAGTTC	TCATTCGAGCTAAAAGCAGA	236
AOS	AY488135	ACAAGGTGGAGAAGAAGGAC	GTCGTTGAGCTTGTTGAACT	260
ACC ox	AY359573	GATCGACTTCTCCAAGCTG	TGTAGAAGATGTCCTCCCAGT	281
Ef 1a	AF136829	GCTTCACGTCCCAGGTC	ATAGGCTTGGTGGGTATCA	212
RPL17	AF034948	CAAAGTCTCGCCACTCCA	CGTCCGTGAGCACGGTA	190

2011), were significantly upregulated as early as 3 h after initiation of ECB feeding, and remained elevated over the entire 24 h time course (Fig. 1a). Induction of *ASsub2* preceded the induction of anthranilate. Anthranilate levels increased approximately 30-fold at 12 h and 100-fold at 24 h as compared to control tissue (Fig. 1b). Indole also accumulated in ECB damaged tissue, but levels peaked at 3 h and returned to basal levels by 12 h (Fig. 1c). The benzoxazinoid profile was altered significantly by ECB stem herbivory at 24 h (Fig. 1d). Levels of DIMBOA-Glc decreased three-fold in ECB damaged tissue as compared to control tissue. In wounded and ECB damaged tissues, an additional benzoxazinoid, HDMBOA-Glc, was detected. Levels of HDMBOA-Glc were 2.2-fold greater in ECB damaged tissue as compared to wound tissue. Overall total benzoxazinoid levels increased from 765 ng g^{-1} FW in control tissue to 1081 and 1350 ng g^{-1} FW in wounded and ECB damaged tissue, respectively.

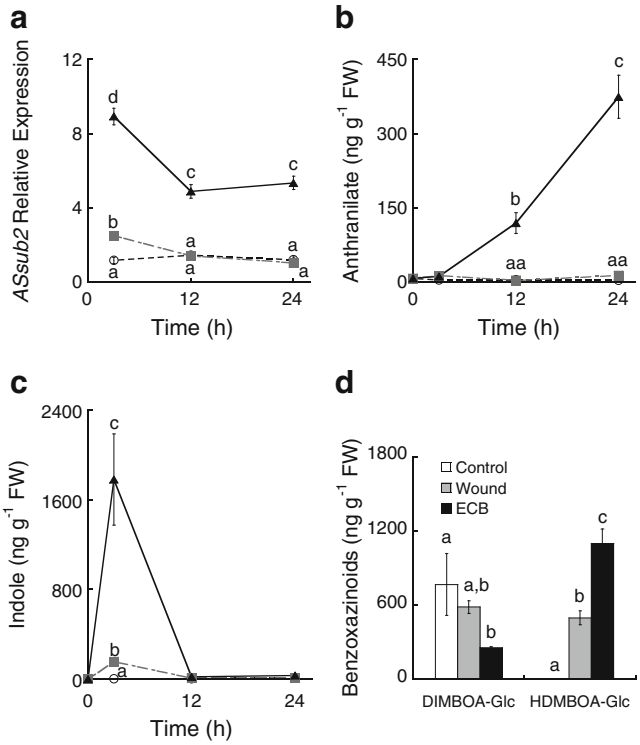


Fig. 1 European corn borer (ECB) herbivory in maize stems induces changes in benzoxazinoids and their precursors. **a** Average relative expression of the *anthranilate synthase subunit 2* (*ASsub2*) gene over time in maize stems that were untreated (○), wounded (■), or infested with ECB (▲;*N*=3, ±SEM). Average concentrations of (**b**) anthranilate and (**c**) indole over time (*N*=3, ±SEM). **d** Comparison of average concentrations of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (DIMBOA-Glc) and 2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (HDMBOA-Glc) at 24 h between control (white bars), wounded (gray bars), and ECB infested stems (black bars; *N*=6, ±SEM). Different letters (a-c) represent significant differences ($P<0.01$ for all ANOVAS; $P<0.05$ for Tukey test corrections for multiple comparisons)

Expression of *An2*, a gene encoding an *ent*-copalyl diphosphate synthase associated with kauralexin synthesis (Schmelz et al. 2011), is significantly upregulated in both wounded and ECB treated tissues as compared to control tissues (Fig. 2a). At 24 h, *An2* transcript levels displayed 9.8- and 31-fold increases in wounded and ECB damaged tissues, respectively. When comparing concentrations at 24 h, total kauralexin levels displayed 2.3-fold greater increases in ECB damaged tissue compared to wounded tissue alone (Fig. 2b).

JA and ET Levels Are Upregulated in Response to ECB Herbivory To examine defense signaling in maize stems, we analyzed ECB induction of the phytohormones JA and ET and transcript levels of their key biosynthetic enzymes. Allene oxide synthase (AOS) is the first dedicated enzyme in the biosynthesis of JA (Wasternack and Kombrink 2010), and the expression of the *AOS* gene is significantly upregulated in response to wounding, and to an even greater extent, ECB feeding as early as 3 h (Fig. 3a). *AOS* transcript levels remained elevated at 12 h in ECB damaged tissues before decreasing at 24 h. In wounded tissue, *AOS* expression levels sharply declined at 12 h and remained low throughout the remainder of the time course. Even though *AOS* increased in both treatments, JA levels significantly increased only in ECB damaged tissue (Fig. 3b). JA levels peaked at 3 h in ECB damaged tissue with a 6.8-fold and 3.7-fold increase as compared to control and wounded tissue, respectively. JA levels remained elevated in ECB damaged tissue, although levels did slightly decrease at 12 h.

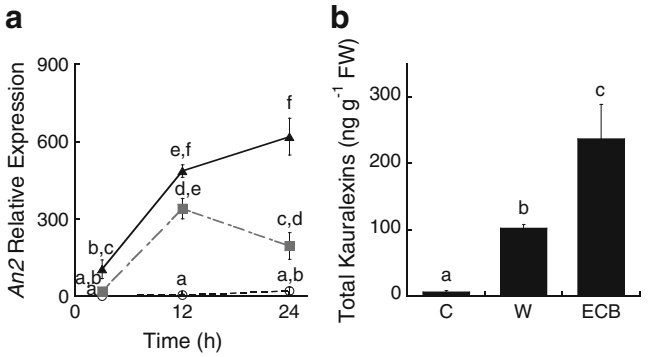


Fig. 2 European corn borer (ECB) herbivory elicits an accumulation of total kauralexins. **a** Average relative expression of the *anther ear 2* (*An2*) gene over time in maize stems that were left undamaged (○), wounded (■), or infested with ECB (▲; N=3, ±SEM). **b** Comparison of the average concentration of total kauralexins at 24 h between control (C), wounded (W), and ECB infested stems (N=6, ±SEM). Different letters (a–f) represent significant differences (P<0.01 for all ANOVAS; P<0.05 for Tukey test corrections for multiple comparisons)

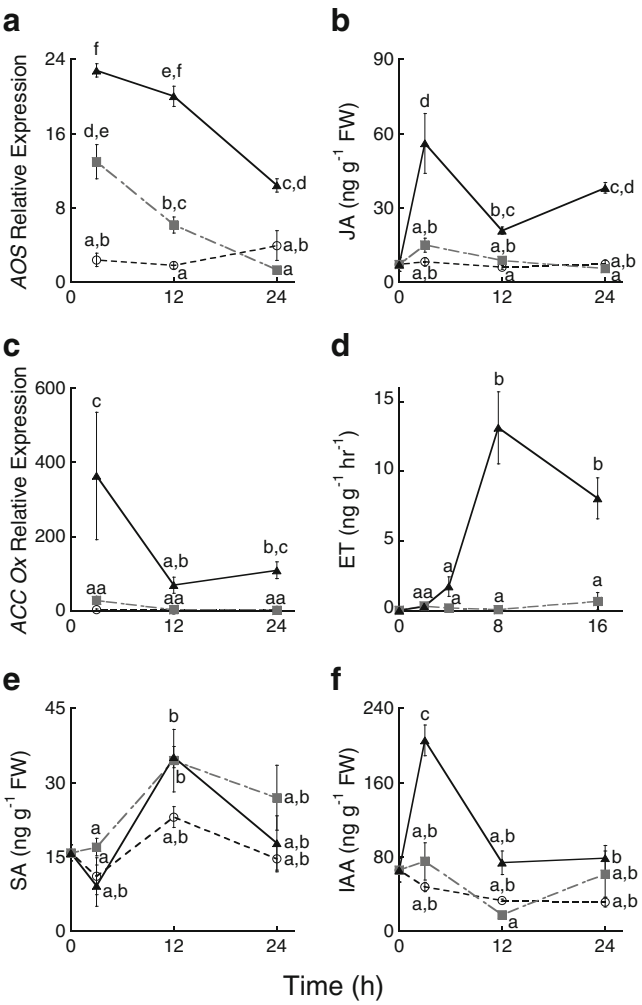


Fig. 3 Changes in phytohormones in response to European corn borer (ECB) herbivory. **a** Average relative expression of the *allene oxide synthase* (*AOS*) gene over time in control (○), wounded (■), and ECB damaged maize stems (▲). **b** Average concentration of jasmonic acid (JA) over time. **c** Average relative expression of the *1-aminocyclopropane-1-carboxylate oxidase* (*ACC Ox*) gene over time. Average concentrations of (**d**) ethylene (ET), (**e**) salicylic acid (SA), and (**f**) indole-3-acetic acid (IAA) over time. Within each panel, different letters (a–f) represent significant differences (P<0.01 for all ANOVAS; P<0.05 for Tukey test corrections for multiple comparisons). For all graphs; N=3, ±SEM

Transcript levels of the gene encoding the ethylene biosynthetic enzyme, 1-aminocyclopropane-1-carboxylic acid oxidase (ACC Ox), also were significantly higher in ECB damaged tissue. At 3 h, transcript levels were 107- and 13-fold greater than control and wound samples, respectively (Fig. 3c). Even though levels of the *ACC Ox* transcript significantly decreased in ECB damaged tissues after 3 h, the average transcript levels remained consistently higher than control and wounded samples over the 24 h time course. Ethylene emission in ECB damaged stem tissue peaked at 8 h (Fig. 3d). Compared to the time zero sample, there was no increase in ET emission detected in wounded tissue at any time point.

In addition to JA and ethylene, salicylic acid (SA), and indole-3-acetic acid (IAA) levels were measured in control, wound, and ECB damaged tissues. There was no significant difference observed for SA levels when comparing the three treatments at 3, 12, and 24 h (Fig. 3e). There was, however, a significant increase in IAA detected in ECB damaged tissues at 3 h (Fig. 3f). As compared to control and wounded samples, IAA was induced 4.3- and 2.7-fold, respectively. IAA levels then decreased to basal levels at 12 h.

JA and ET Regulate Changes in Benzoxazinoids To test whether JA and ET may have a role in regulating ECB-induced responses in stems, JA and the ET precursor, (ACC), were applied to mechanically wounded maize stems, and levels of benzoxazinoids and kauralexins were measured. Similar to the trend observed with ECB feeding, exogenous application of JA alone or in combination with ACC resulted in decreases of DIMBOA-Glc as compared to control and wounded plants (Fig. 4). However, only the combination of JA and ACC resulted in significant increases in HDMBOA-Glc above wounding alone as evidenced by 3.2-fold higher concentrations. Treatment with ACC did not result in any changes that were significantly different from wounding alone. HDMBOA-Glc was not detected in unwounded control plants.

Treating stem internode tissues exogenously with JA, ACC, or the combination of JA and ACC did not significantly affect levels of kauralexins (Supplemental Fig. 2). However, the concentration of total kauralexins was significantly increased in wounded tissue as compared to control tissue.

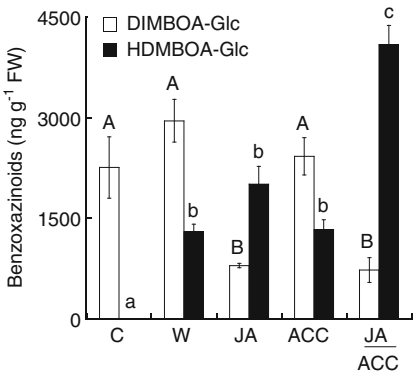


Fig. 4 In maize stem internode tissue, treating with a combination of jasmonic acid (JA) and the ethylene (ET) precursor, aminocyclopropane-1-carboxylic acid (ACC), elicits changes in benzoxazinoids similar to those observed with European corn borer (ECB) feeding. Comparison of average concentrations of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (DIMBOA-Glc, white bars) and 2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (HDMBOA-Glc, black bars) at 24 h between control (C), wounded (W), JA, ACC, and JA and ACC treated stems (N=5, ±SEM). Upper case letters (A–B) represent significant differences in DIMBOA-Glc levels and lower case letter (a–c) represent significant differences in HDMBOA-Glc levels (P<0.01 for all ANOVAS; P<0.05 for Tukey test corrections for multiple comparisons)

ECB Growth not Affected by Chemical Defenses in Maize Stems To determine if ECB-induced maize defenses could effectively inhibit the growth of ECB, final instars were allowed to feed on stem tissues that had previously experienced no treatment, wounding, or ECB damage for 24 h. After 24 h of feeding on these tissues, no significant differences were observed between the percent weight gains (Fig. 5a) or tissue consumption (Fig. 5b) of ECB feeding on control, wounded, and ECB damaged stem tissues. This suggests that ECB larvae are largely unaffected by short-term changes in maize defenses.

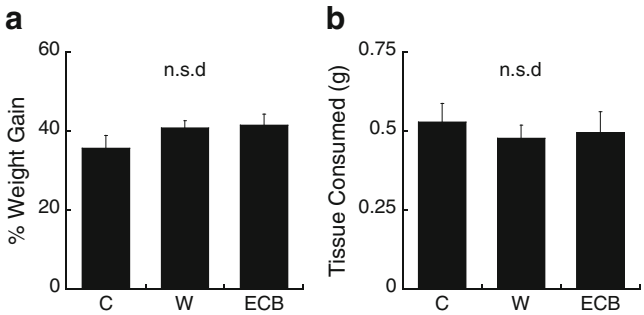


Fig. 5 European corn borer (ECB) growth is not affected by the presence of maize chemical defenses. **a** Average percent weight gain for ECB fed for 24 hrs on tissues previously subjected to either no treatment (C), wounding (W), or ECB stem herbivory (ECB). **b** Average amount of tissue consumed by ECB over the 24 hr feeding period. Not statistically different (n.s.d) indicates P>0.05 for all ANOVAs. For both graphs; N=13, ±SEM

Discussion

ECB are one of the most important insect pests of maize (Ostlie et al. 1997); however, surprisingly little is known about ECB-induced chemical defenses in maize stems. For example, DIMBOA is a constitutively expressed phytoanticipan known to function in defense against ECB herbivory in leaves (McMullen et al. 2009; Niemeyer 2009), but the dynamics of benzoxazinoids in maize stems in response to ECB herbivory has not been well studied. For leaves of young maize seedlings, biotic cues, including fungal infection and insect herbivory, have been shown to promote the synthesis of the benzoxazinoid, HDMBOA-Glc with a corresponding decrease in DIMBOA-Glc levels (Oikawa et al. 2001, 2004). In this study, we observed a similar pattern, thus demonstrating that insect inducible benzoxazinoid production continues to occur in mature plants and importantly in stem tissues.

In an effort to understand how defenses like benzoxazinoids are regulated in response to ECB stem herbivory, levels of plant hormones were analyzed in control, wounded, and ECB damaged tissues. Significant increases were observed for JA and ET in response to ECB feeding indicating that these two hormones may be involved in regulating the expression of maize stem defenses. Applying JA and the ET precursor, ACC, concurrently to stem internode tissue mimicked the HDMBOA-Glc accumulation observed with ECB feeding, and provided additional support for this hypothesis. While similar, these results appear to differ with the plant hormonal regulation of maize benzoxazinoids in leaves where the application of JA alone to maize leaves triggers HDMBOA-Glc accumulation (Oikawa et al. 2001). It is possible that different signaling mechanisms may be utilized in different plant tissues. A similar discrepancy was observed when analyzing total kauralexins, a class of diterpene phytoalexins that have ECB anti-feedant properties (Schmelz et al. 2011). Concentrations of kauralexins were upregulated significantly when young, actively growing stem tissue was treated with a combination of JA and ethephon or JA and ACC (Schmelz et al. 2011). In the current study, when stem internode tissue (tissue that is not actively growing or differentiating) was treated with JA, ACC, or the combination of JA and ACC, the levels of kauralexins were not significantly altered. However, total kauralexins did increase in stem internode tissue when subjected to ECB herbivory, indicating a requirement for additional, currently unknown stimuli beyond JA and ET. Thus, the hormonal regulation of benzoxazinoids and kauralexins appears to be tissue dependent, and although both classes of defensive compounds are induced in response to ECB stem herbivory, they appear to require different combinations of phytohormone signals within the same tissue.

In addition to JA and ET, the phytohormone IAA also may be involved in regulating the induction of defensive com-

pounds in maize stems. IAA generally is considered an antagonist of JA, and it can suppress the induction of defensive compounds such as protease inhibitors and nicotine (Baldwin et al. 1997; Saniewski et al. 2002). Generally, IAA decreases in response to wounding (Thornburg and Li 1991, Schmelz et al. 2003b), however, it rapidly and significantly increased in maize stems in response to ECB herbivory. We are currently in the process of trying to understand how IAA may function in the maize defense response.

Both benzoxazinoids and kauralexins have anti-insect properties (McMullen et al. 2009; Niemeyer 2009; Schmelz et al. 2011), yet it is unknown how these compounds function *in planta* against ECB stem boring. DIMBOA functions in defense against ECB feeding on maize leaves (McMullen et al. 2009; Niemeyer 2009), but the levels of DIMBOA are much lower in stem tissue (Niemeyer 2009), and the levels decrease even further with ECB stem herbivory. As compared to DIMBOA levels in young leaves, the low levels in stems likely fail to afford sufficient protection. We demonstrate that ECB and the concurrent application of JA and ACC induces HDMBOA-Glc in stems, but far less is known regarding the activity of this compound, in part because benzoxazinoids such as HDMBOA are highly unstable (Woodward et al. 1978; Grambow et al. 1986). There are studies suggesting that HDMBOA can have anti-insect activity (Hedin et al. 1993; Cambier et al. 2001), but the effect of HDMBOA on ECB has not yet been investigated. Unlike HDMBOA, the effect of kauralexins on ECB has been studied, and these compounds have ECB anti-feedant activity when applied to stem sections at 50 $\mu g\ \mu g^{-1}$ FW (Schmelz et al. 2011). This treatment is consistent with the concentration of kauralexins induced by fungal pathogens (Schmelz et al. 2011), but it is much higher than the concentration in ECB damaged stem sections that occurs at 24 h (0.2 $\mu g\ \mu L^{-1}$). However, similar to benzoxazinoids, kauralexins can be unstable and, therefore, the biological activity detected in the ECB choice feeding assay (Schmelz et al. 2011) may be a conservative estimate due to degradation.

To minimize the degradation of benzoxazinoids and kauralexins, we fed ECB previously treated stem tissue instead of artificial diet supplemented with purified products. In addition to known phytochemicals, the tissue would have an array of other inducible defenses present, which could potentially function synergistically in defense against ECB herbivory. Inducible plant defenses often effectively increase resistance against insect feeding by reducing insect growth and decreasing fecundity (Agrawal 1999; Bruinsma et al. 2007; Abe et al. 2008, 2009; Stout et al. 2009; Brunissen et al. 2010). In contrast, plants that are deficient in JA signaling and have a compromised defense response are far more susceptible to insect feeding (Halitschke and Baldwin 2003; Abe et al. 2008, 2009; Scott et al. 2010). Although maize stems accumulated significant quantities of defensive compounds 24 h after the initiation of ECB feeding, the growth of ECB was not affected. It is possible that long-term exposure to these defenses beginning at an earlier developmental stage could have a significant impact on the growth and development of ECB; however, our current findings suggest that ECB may have mechanisms to cope with maize chemical defenses. In the related Asian corn borer [*Ostrinia furnacalis* (Guenée)], larvae can withstand low levels of hydroxamic acids, most likely by enzymatically degrading them (Kojima et al. 2010). In contrast, the rice armyworm [*Mythimna separata* (Walker)] glycosylates benzoxazinoids making them less reactive (Sasai et al. 2009). ECB may have similar detoxification strategies, or alternatively, defenses other than the ones studied here may be more important in stem resistance against ECB feeding in the stem (McMullen et al. 2009). For example, transgenic maize expressing a wheat oxalate oxidase gene had decreased DIMBOA levels, but increased resistance that was positively correlated to increases in concentrations of soluble phenolic acids (Mao et al. 2007). Although the defenses induced by ECB in maize stems appear not to be effective against ECB, they may function against subsequent threats by invading fungal pathogens. In addition to anti-insect activity, both benzoxazinoids and kauralexins have been shown to have anti-fungal properties (Niemeyer 2009; Schmelz et al. 2011).

The current study provides a basis for understanding inducible defenses in maize stems and in part, how these defenses are regulated. ECB growth does not appear to be affected by these defenses, leading to many interesting questions as to how ECB subverts or tolerates maize defenses.

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plant's natural development. Thus, ontogenetic trajectories in plant constitutive defenses can interact with herbivore-induced defenses leading to changes in the occurrence and strength of induction in unpredictable ways. This question is of particular interest for annual and/or short-lived perennial plant species, whose responses to single or multiple damage events over a growing season are likely to interact with ontogenetic constraints in affecting a plant's ability to respond to herbivory. Yet, empirical tests that assess the combined role of multiple damage events and age-dependent constraints on the ability of plants to induce defenses are scarce.

Constitutive defenses have been shown to vary as a function of plant development, with defensive traits increasing or decreasing over time as resource allocation constraints and plant functional priorities change (Boege and Marquis, 2005). Furthermore, dissimilar ontogenetic trajectories in constitutive chemical defenses have been reported among plant tissues, such as between above- and below-ground tissues (Williams and Ellis, 1989; Bellostas et al., 2007). These ontogenetic trajectories can encompass small to large qualitative and quantitative changes in defensive traits, which may in turn influence the identity of herbivores associated with different plant developmental stages (Waltz and Whitham, 1997; Thomas et al., 2010). Despite the increasing interest in documenting developmental variation in constitutive defenses, the role of plant age in explaining herbivore induced defenses within and among tissues continues to be largely unexplored. In those systems where age-dependent induction of defenses was assessed, however, the ability of plants to resist or tolerate herbivory has been shown to strongly depend on plant developmental stage (Van Dam et al., 2001; Warner and Cushman, 2002; Boege et al., 2007; Muola et al., 2010).

Physiological and morphological constraints during plant ontogeny can lead to strong age-dependent induced responses to herbivory, with opposite trends being predicted for resistance and tolerance. Induced chemical defenses, usually considered a cost-saving strategy for plants, are associated with scenarios where resource allocation constraints limit constitutive defenses or are dependent on actively growing and differentiating tissues (Karban and Baldwin, 1997; Cipollini et al., 2003). Because younger plant stages often are more susceptible to herbivory, have lower levels of constitutive defenses, and proportionally more undifferentiated and actively growing tissues than older plant stages, the ability of plants to induce defenses is predicted to be greater in younger developmental stages and to lessen as plants mature (Karban and Baldwin, 1997). Alternatively, secondary growth that aims to compensate for lost tissue requires the reallocation of resources stored in undamaged tissues, as well as the enhancement of photosynthetic rates and nutrient uptake from the soil. Therefore, tolerance to herbivory is predicted to increase with plant age due to greater capacity to acquire resources and/or higher probability to have already stored reserves (Strauss and Agrawal, 1999; Haukioja and Koricheva, 2000). While there is some support for such patterns (Boege and Marquis, 2005; Barton and Koricheva, 2010), this is mostly based on comparisons of single herbivore damage events during plant development, and as such, do not reflect potential plant responses due to natural variation in multiple and successive damage events over the course of development.

Sequential damage over a growing season can alter age-dependent induced responses to herbivory in two ways. First, repeated damage events by an herbivore species can induce an enhanced response as compared to a single damage event (e.g., Ruuhola et al., 2007; Roitto et al., 2009), as a result of what has been called 'immunological memory' (Baldwin and Schmelz, 1996). Alternatively, plant responses to a previous damage event may reduce the ability of a plant to defend itself against a later attacker (e.g., Viswanathan et al., 2007; Poelman et al., 2008). This pattern, usually termed 'priority effect', can arise as a consequence of physiological limitations such as negative cross-talk between pathways elicited by different herbivores (Verhage et al., 2010) or because of resource limitations if resources used during an initial response are unavailable for use in later responses. Thus, history of damage over a season or throughout plant development can strongly enhance or diminish expected age-dependent induced responses, potentially altering key plant-herbivore interactions. Furthermore, because allocation patterns and synthesis of chemical defenses may vary within and among tissues over time as a response to plant development and history of damage (Williams and Ellis, 1989; Van Dam et al., 2001; Bellostas et al., 2007; Kaplan et al., 2008), it is essential that we incorporate both above- and below-ground plant responses to current and past damage to fully understand the complexity of factors that shape plant defenses and, as a consequence, plant-mediated herbivore interactions.

Here, by using *Plantago lanceolata* L. (Plantaginaceae) and one of its specialist chewing herbivores, *Junonia coenia* Hübner (Lepidoptera: Nymphalidae), we examined the effect of plant ontogeny (two developmental stages) and history of damage (single and multiple damage events early or late in the season) on plant responses to herbivory in both above- and below-ground tissues. We specifically ask the following questions: 1) Do levels of constitutive defenses, as well as induced defenses and compensatory regrowth following leaf damage by a specialist chewing herbivore, vary with plant age?, 2) For mature plants, does variation in frequency and intensity of herbivory early

during a growing season influence plant induced chemical defenses and compensatory regrowth later in the season?, and 3) To what extent do changes in above-ground plant responses to leaf chewing damage, due to plant age or previous history of damage, translate into changes in below-ground biomass and defenses?

Methods and Materials

Study System Plantago lanceolata L., narrowleaf or ribwort plantain, (Plantaginaceae) is a common short-lived weed (annual or facultative perennial) introduced to North America from Eurasia ca. 200 years ago (Cavers et al., 1980). This species forms natural populations with diverse age structures (Shefferson and Roach, 2010) and thus, herbivore communities are naturally exposed to a wide diversity of host age classes in wild populations. *Plantago lanceolata* produces iridoid glycosides (IGs) (Rønsted et al., 2000) as its primary allelochemicals that influence feeding preferences of generalist and specialist rhizophagous and folivorous herbivores. For example, several laboratory and field experiments have shown that specialist lepidopterans use iridoid glycosides as oviposition and feeding stimulants (Bowers, 1983, 1984; Talsma et al., 2008), and that larvae of these herbivores grow better on diets with higher concentrations of IGs (e.g., Bowers and Puttick, 1989; Harvey et al., 2005; Saastamoinen et al., 2007). In contrast, generalist herbivores and pathogenic fungi that damage below- or above-ground tissues usually are deterred by high concentrations of IGs (Bowers and Puttick, 1989; Strohmeyer et al., 1998; Biere et al., 2004; De Deyn et al., 2004; Harvey et al., 2005; Wurst and van der Putten, 2007). *Plantago lanceolata* produces primarily two IGs, aucubin and catalpol, and amounts of these compounds may be as high as 10–12% dry weight (Bowers and Stamp, 1993). Because aucubin is the biosynthetic precursor of the more toxic catalpol (Rønsted et al., 2000), high relative concentrations of catalpol indicate not only a greater biosynthetic investment of the plant, but also may be important in determining how generalist and specialist herbivores respond to the plant. Finally, constitutive concentrations of IGs have been shown to increase non-linearly during plant ontogeny (Fuchs and Bowers, 2004; Quintero and Bowers, 2011) leading to significant seasonal variation. The variation in constitutive defenses in both above- and below-ground tissues can be as much as an order of magnitude, even during relatively short periods of time (Bowers and Stamp, 1993; Fuchs and Bowers, 2004; Barton, 2007; Quintero and Bowers, 2011), which can lead to significant variation in a plant's ability to induce chemical defenses within and among tissues.

The common Buckeye butterfly (*Junonia coenia* Hübner, Nymphalidae) is a New World butterfly that can have one to three broods per year under temperate conditions, or more in the tropics where it can breed year-round (http://www.nearctica.com/butter/plate17/Jcoenia.htm). The larvae feed on members of four plant families: Cornaceae, Plantaginaceae, Scrophulariaceae, and Verbenaceae, and this species is considered a specialist on plants containing iridoid glycosides (Bowers, 1984). In *P. lanceolata*, the two major IGs, aucubin and catalpol, serve as feeding and oviposition stimulants for Buckeyes (Bowers, 1984; Pereyra and Bowers, 1988). In addition, Buckeye larvae not only sequester IGs from their hosts, but larval IG content also is positively correlated with plant IG content (Camara, 1997; Prudic et al., 2005); thus, caterpillar performance and predation risk can strongly depend on levels of host plant defenses (Dyer and Bowers, 1996; Theodoratus and Bowers, 1999). Nevertheless, high concentrations of IGs also may have some detrimental effects by decreasing larval performance (Adler et al., 1995; Camara, 1997) and/or by enhancing larval susceptibility to parasitoid attack (Smilanich et al., 2009).

Plant and Caterpillar Colony Maintenance The study was performed at the University of Colorado during spring-summer 2006. Seeds were collected from >20 maternal *P. lanceolata* plants from several populations in Boulder County, Colorado, and mixed before the experiment started. Seedlings were germinated in Fafard mix on April 26th and transplanted after 15 d to a 1.5 L plastic pots containing growth medium of equal parts Metro Mix 350™, sterilized sand, and turface. These extra-deep pots were used in order to ensure natural growth of root as well as shoot tissues and to prevent pot-binding, which can affect both compensatory regrowth and plant secondary metabolite production (Karban and Baldwin, 1997). All seedlings used were selected to be equivalent in size and number of leaves at the time of transplanting. Buckeye larvae used were from a laboratory colony reared at the University of Colorado at Boulder. Larvae were fed on a mix of *P. lanceolata* leaves and kept in growth chambers with a photoperiod of 14 h day: 10 night, and day-night temperatures of 27°C /22°C.

Plant Age and Responses to Herbivory To assess the effect of plant age on plant induced responses to herbivory, juvenile and mature *P. lanceolata* plants were damaged by *J. coenia* caterpillars when plants were 9- and 14-wk-old, respectively. We chose 5 wk as a meaningful period between age classes because this is a good approximation of the average generation time of *J. coenia* (personal observation). In addition, 9- and 14-wk-old age classes were *a priori* selected to represent two distinct but still comparable developmental stages. These stages are

comparable in the sense that both are already independent from reserves stored in the seed, contain several new and intermediate leaves but lack mature leaves, and show no significant variation in terms of nutritional quality and physical defenses (i.e., <1% difference in nitrogen content, <4% difference in water content, and <10% difference in leaf toughness; unpublished data). However, while 9-wk-old plants, designated juvenile plants, still have a high relative growth rate and lower constitutive levels of IGs, 14-wk-old plants, designated mature plants, usually are reaching the onset of flowering, have started to decrease their vegetative growth rate, and constitutive defenses are often double those of juvenile stages (see "Results").

At both age classes, 40 previously undamaged plants were randomly assigned to one of two treatments: no herbivore (C: control) or damage by *J. coenia* caterpillars (H: herbivore). In order to account for potential differences between treatments before the experiment started, initial plant size of all plants at the beginning of the herbivory treatments was measured as the product of the length of the longest leaf and the total number of leaves, which provides a good index of plant size (Jarzomski et al., 2000). To confine caterpillars on the plants, control and treatment plants were caged using mesh bags constructed of Remay™. For juvenile plants, two newly molted 4th instars per plant were placed in the center of the plants in the herbivory treatment. For mature plants, we applied from four to seven 4th instars per plant in order to reach approximately 50% damage during an equivalent period of time. In both cases, 2 d after larvae were placed on plants or when larvae had eaten approximately 50% leaf tissue, all caterpillars and mesh bags were removed. Since induction of IGs was estimated to reach its highest point 6 d after damage (Fuchs and Bowers, 2004), to test for short-term plant induced defenses as a function of plant age, all plants per treatment per age class were harvested 7 d after herbivore removal.

Harvested plants were separated into above- and below-ground tissues. Above-ground tissues consisted of new and intermediate leaf age classes (see Bowers and Stamp, 1993) for both juvenile and mature stages. Thus, although we did not asses variation in plant defenses within different leaf age classes, we kept equal representation of leaf ages classes across plant developmental stages. Root tissues were washed until roots were totally clean from remaining soil material. Due to time constraints, root tissues from only half of the plants were processed (N=10 per treatment). Harvested tissues were weighed fresh, oven-dried at 50°C for 48 h to a constant mass, and weighed again to the nearest hundredth of a gram. By the time plants reached their mature stage (14-wk-old plants), a few plants per treatment had started flowering. However, because inflorescence biomass was usually <10% of the total above-ground biomass and because the proportion of plants flowering did not vary among treatments (P>0.05), we added data obtained from reproductive tissue data to leaf data in order to report overall above-ground (i.e., shoot) values for biomass and chemical defenses. Furthermore, we ran all analyses for the complete data set as well as separately, using just flowering or non-flowering individuals; but because trends were not significantly different (data not shown), we present here the results for the complete data set.

To assess variation in concentrations of IGs, dried shoots and roots were ground into a fine powder, and subsamples of 25 to 50 mg were processed for IG extraction and analyzed by gas chromatography following previously described methods (Bowers and Stamp, 1993; Jarzomski et al., 2000). Briefly, samples were extracted overnight in 95% methanol, and then partitioned between water and ether to remove hydrophobic compounds, using phenyl-β-D-glucose (PBG) as our internal standard. An aliquot of the solution was derivatized with Tri-Sil-Z™ (Pierce Chemical Company) and injected into an HP 7890A gas chromatograph (Agilent Technology) using an Agilent DB-1 column (30 m, 0.320 mm, 0.25 μm particle size). Amounts of aucubin and catalpol were quantified using ChemStation B-03-01 software, and they are presented as percent dry weight for comparative purposes (e.g., Fuchs and Bowers, 2004; Barton, 2007; Wurst and van der Putten, 2007).

Independent sample *t-tests* were used to test for possible differences in initial plant size between treatments of 9- or 14-wk-old plants before the herbivory treatments were applied. Plant responses to herbivory (C vs. H) at juvenile or mature plant stages were assessed as changes in shoot and root biomass (dry mass), total concentration of IGs (% dry weight), and proportion of catalpol/total IGs. We used a series of two-way analyses of variance (ANOVA) with age and treatment as the main effects and an interaction term included. Iridoid glycoside concentration data were arcsine-square root transformed for statistical analyses, but biomass data did not require transformation. Separate two-way ANOVAs were performed for shoot and root tissues since root tissues were available for only half of the plants (N=10 for roots and N=20 for shoots for each herbivory : age treatment combination). When the herbivory treatment effect was significant, we followed with single degree of freedom contrasts to assess differences between treatments (C vs. H) at the two different age classes.

History of Damage and Plant Responses to Herbivory To determine how variation in the frequency and intensity of damage might affect plant performance and IG concentration, plants received a combination of damage and non-

damage events early and later in the season. First, 80 juvenile plants (i.e., 9-wk-old) were divided in two treatments: control and herbivory (N=40 for each) as described above. Five weeks following the first damage event to juvenile plants (50% damage), 14-wk-old plants (N=40 undamaged and 40 previously damaged plants) were exposed to a second generation of Buckeye caterpillars (50% damage). Thus, for this experiment there were 4 treatments: 1) plants that were never damaged (control-control: CC); 2) plants damaged by the first generation of Buckeyes but not by the second generation (herbivory-control: HC); 3) plants not damaged by the first generation of Buckeyes but damaged by the second generation (control-herbivory: CH); and 4) plants damaged by both generations of Buckeyes (herbivory-herbivory: HH), with 20 replicates per treatment. As before, when caterpillars achieved 50% damage during both first and second damage events, all caterpillars and exclusion cages from control and treatment plants were removed. Seven days after the last damage event, all plants per treatment were harvested and separated into shoot and root tissues to assess differences in plant biomass and induced defenses as explained above.

To assess the effect of previous herbivory on subsequent plant responses to damage, 1 wk after the second generation of damage, differences in biomass, total concentration of iridoid glycosides, and proportion of catalpol/total IGs in shoot and root tissues were evaluated using one-way ANOVAs. Because sample sizes were different between root and shoot tissues (see above) these data were analyzed separately. In all cases, when significant effects were detected by ANOVAs, we followed up with *a priori* contrasts comparing (1) control vs. herbivory treatments (CC vs. HC + CH + HH), (2) plant responses to damage received early vs. late in the season (HC vs. CH), and (3) mature plant responses to a single herbivory event vs. multiple events (CH vs. HH).

Results

Plant Age and Responses to Herbivory At the beginning of the experiment, above-ground size of plants in the two herbivory treatments (C and H) were not significantly different from each other for either juvenile plants ($t_{1,38}$ −0.07; P=0.51), or for mature plants ($t_{1,38}$ 5.6; P=0.13). Based on the subset of plants harvested the same day that treatments started, juvenile plants at the beginning of the experiment had, on average, 12 leaves, their average above-ground biomass (dry weight) was 0.5±0.04 g, and their below-ground biomass was 0.13±0.01 g. Mature plants had, on average, 56 leaves, their above-ground biomass was 6.05± 0.29 g, and their below-ground biomass was 4.14±0.24 g (dry weight).

One week following the first generation of damage, above-ground plant responses to herbivory were strongly influenced by plant age (Table 1, Fig. 1). Above-ground biomass was significantly higher in control plants and older plants, but no interaction was observed (Table 1). Thus, 1 wk following 50% leaf tissue removal, damaged plants in both age classes were unable to compensate for the lost tissue (Fig. 1a), with damaged plants being on average 46% and 24% smaller than control plants for juvenile and mature plants, respectively (juvenile: $F_{1,76}$ 6.5, P=0.013; mature: $F_{1,76}$ 24.5, P<0.001). Therefore, although there was no significant interaction between age and herbivory, mature plants were better than juvenile plants at compensating for the lost tissue 1 wk following damage. In turn, induced defenses after herbivory in above-ground tissues were observed only for proportion of catalpol but not for total levels of IGs (Table 1). Total levels IGs were 16 times greater in mature plants when compared to juvenile plants; but they did not significantly vary between control and herbivory treatments in either age class (Fig. 1b). In contrast, the proportion of catalpol not only varied with plant age, showing an average decrease of 47% from

Table 1 Summary of statistical results (anova) comparing *Plantago lanceolata* responses in shoot and root tissues one week after herbivory treatments[1] for juvenile and mature plants

	Shoot tissues							Root tissues						
		Biomass		Total IGs		Prop Catalpol			Biomass		Total IGs		Prop Catalpol	
	df	*F*	*P*	*F*	*P*	*F*	*P*	df	*F*	*P*	*F*	*P*	*F*	*P*
Plant age	1	452.1	<0.001	541.7	<0.001	43.3	<0.001	1	124.1	<0.001	20.2	<0.001	12.5	<0.001
Herbivory	1	28.2	<0.001	0.2	0.67	17.0	<0.001	1	1.9	0.17	0.01	0.95	1.1	0.30
Age*Herbivory	1	2.9	0.095	0.06	0.80	10.4	0.002	1	0.2	0.63	0.38	0.54	3.4	0.07
Error	76							36						

[1] Plant responses to first damage event (50% leaf tissue removal) was assessed as changes in biomass (dry mass), total concentration of iridoid glycosides (% dry weight IGs), and proportion of catalpol/total IGs

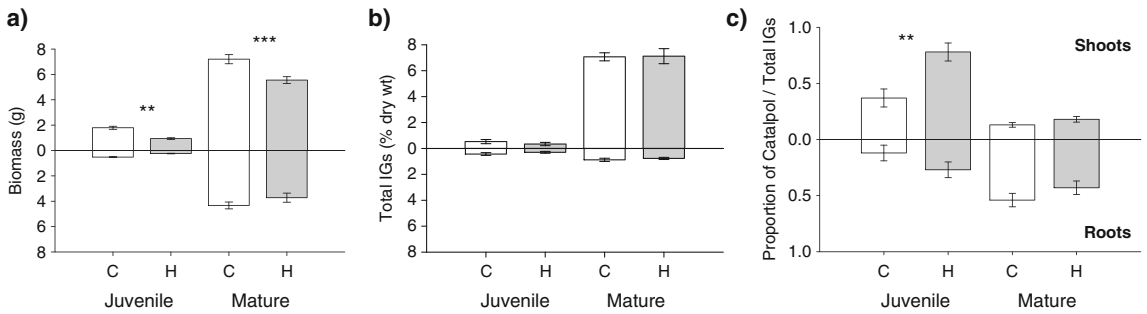


Fig. 1 Plant responses to herbivory in *Plantago lanceolata*, above- and below-ground **a)** biomass, **b)** total concentration of iridoid glycosides (IGs), and **c)** proportion of catalpol/total IGs for control (C; white bars) and herbivore damaged plants (H; gray bars) during juvenile or mature plant stages. Original data were arcsine-square root transformed for statistical analyses, percentage data are shown for illustrative purposes only. Asterisks represent mean group differences among treatments as tested by single degree of freedom contrasts. Significance is displayed as $P<0.001$ (***), $P<0.01$ (**), $P<0.05$ (*)

juvenile to mature plants, but also the ability of a plant to induce above-ground chemical defenses depended on host plant age (Table 1). Single degree of freedom contrasts showed that 1 wk following 50% tissue removal, damaged juvenile plants showed a 45% increase in their levels of catalpol/total IGs ($F_{1,76}$ 9.7, P=0.003), while mature plants did not differ from control plants in the proportion of catalpol (Fig. 1c) ($F_{1,76}$ 0.3, P=0.58). This difference is illustrated by a significant interaction between plant age and treatment in above-ground proportion of catalpol (Table 1).

In contrast, although below-ground tissues followed similar patterns to the ones seen for above-ground tissues, ANOVA results showed only a significant effect of plant age on root biomass and defenses; but leaf damage by Buckeye caterpillars did not translate into changes in root biomass or chemical defenses between control and herbivory treatments one week following damage (Table 1, Fig. 1).

History of Damage and Plant Responses to Herbivory One week following the second generation of damage by Buckeye caterpillars, ANOVA results showed that herbivory treatment had a negative effect on shoot biomass ($F_{3,76}$=21.46, P<0.001) and a nearly significant negative effect on root biomass ($F_{3,36}$ 2.77, P=0.056) (Fig. 2a). Therefore, compensatory regrowth was not achieved 1 wk or even 5 wk following herbivore damage. Single degree of freedom contrasts showed that a decrease in shoot biomass was observed between undamaged control plants and damaged plants (CC vs. HC + CH + HH): $F_{1,76}$ 44.9, P<0.001), as well as between mature plants damaged once vs. those damaged twice (CH vs. HH: $F_{1,76}$ 18.7, P<0.001) (Fig. 2a). However, no difference in shoot biomass was observed between plants with one single damage event early vs. late in the season (HC vs. CH: $F_{1,76}$ 2.1, P=0.15) (Fig. 2a).

In contrast, we did not observe any significant changes in plant IGs as a response to different histories of damage: production of total IGs and proportion of catalpol/total IGs did not vary with history of damage either for above-ground tissues ($F_{3,76}$=0.99, P=0.40 and $F_{3,76}$=2.39, P=0.08, respectively) or below-ground tissues ($F_{3,36}$=0.13, P=0.94 and $F_{3,36}$=0.59, P=0.63, respectively). Thus, mature plants that

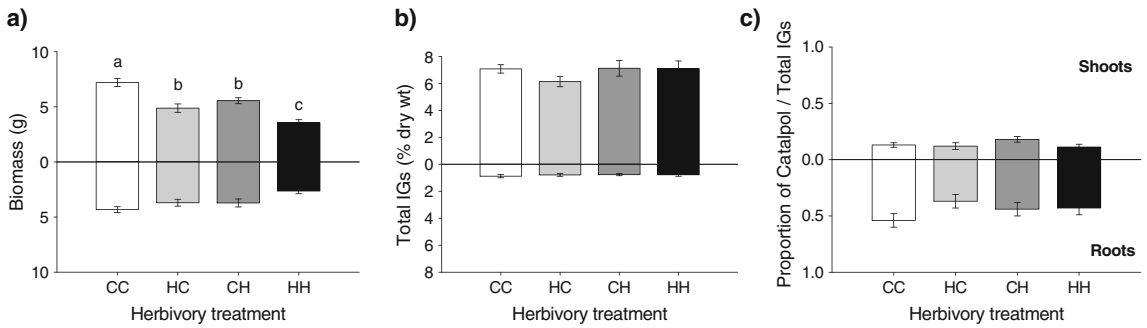


Fig. 2 Effects of herbivory on *Plantago lanceolata*, above- and below-ground **a)** biomass, **b)** total concentration of iridoid glycosides (IGs), and **c)** proportion of catalpol/total IGs 1 wk after second generation of damage. Bars represent the combination of first and second damage events, leading to four treatments: 1) plants that were never damaged (CC); 2) plants damaged by the first generation of Buckeyes but not by the second generation (HC); 3) plants not damaged by the first generation of Buckeyes but damaged by the second generation (CH); and 4) plants damaged by both generations of Buckeyes (HH), with N=20 replicates per treatment. Original data were arcsine-square root transformed for statistical analyses, percentage data are shown for illustrative purposes only. Lowercase letters represent mean group differences among treatments as tested by Bonferroni post-hoc tests (P<0.05)

varied in intensity and frequency of herbivory, had similar levels of defenses across all four damage treatments, and induction of defenses was not observed (Fig. 2b, c).

Discussion

This study revealed novel patterns regarding the extent to which plant age and history of damage over a growing season can modify *Plantago lanceolata*'s ability to respond to herbivory. Most notably, we showed that while constitutive concentration of IGs markedly increased as plants matured, plant ability to induce chemical defenses was limited to juvenile, but not mature, plant stages. In addition, induced defenses observed 7 d following herbivory in juvenile plants disappeared five weeks after the first herbivory event, and mature *P. lanceolata* plants that varied considerably in the absolute amount and frequency of damage received over 5 wk, did not significantly differ in their levels of chemical defenses. Finally, contrary to what we expected, we did not observe changes in below-ground tissues' defenses or biomass a week following 50% removal of above-ground tissues at either age class or history of damage. Together, these results suggest that in *Plantago lanceolata* and perhaps other systems, ontogenetic trajectories in plant growth and defenses can play a stronger role in mediating plant-herbivore interactions than history of damage.

Plant ontogeny strongly influenced plant defenses in both above- and below-ground tissues. Synthesis and accumulation of constitutive IGs increased 13 times in above-ground tissues and doubled in below-ground tissues from juvenile to mature stages. In addition, plant allocation patterns also varied considerably from almost equal investment in total IG concentrations between shoot and root tissues during the juvenile stage to a large increase in plant total IGs in shoots as compared with roots during the mature stage (i.e., ratio of total levels of IGs between shoot and root tissues varied from roughly 1:1 in juveniles to 8:1 ratio in mature plants) (Fig. 1). Comparable trends in constitutive defenses within and among tissues as plants age have been reported previously for *P. lanceolata* (Darrow and Bowers, 1999; Fuchs and Bowers, 2004; Quintero and Bowers, 2011) as well as for other IG-containing plants (Hogedal and Molgaard, 2000; Beninger et al., 2007; Jamieson and Bowers, 2010). These shifts suggest that, even in the absence of damage, plant palatability can dramatically change in a short period of time with strong implications for plant-herbivore interactions and plant fitness. For instance, even smaller differences in concentration of IGs in *P. lanceolata* as the ones reported here have shown to significantly decrease leaf consumption and growth rate of generalist herbivores and pathogens as iridoid content increases (Biere et al., 2004). As a result, herbivore pressure may be higher on younger developmental stages, decreasing their chances of survival or future reproductive output (e.g., Shefferson and Roach, 2010).

While the role of ontogenetic trajectories in constitutive defenses has received considerable attention, our knowledge regarding the role of plant age in explaining herbivore induced defenses is still limited. A recent meta-analysis that summarized ontogenetic patterns in plant defense traits illustrated that, in the case of herbaceous plants, ability to induce defenses after damage usually decreased with plant age, while no significant differences in tolerance were observed across plant development (Barton and Koricheva, 2010). In accordance with this trend, we show that a week following damage, juvenile *P. lanceolata* plants were significantly more capable of induction (i.e., 45% increase in the proportion of catalpol/total IGs) than mature plants, but no significant differences were observed for compensation to herbivory. Nevertheless, lack of differences in compensatory regrowth between age classes should be interpreted carefully since we only assessed changes in biomass between treatment and control plants a week following 50% damage, and thus, this short-term responses may not reflect long-term compensatory regrowth strategies nor be indicative of potential fitness consequences. In terms of induction, because the defensive properties of iridoid glycosides have been well documented (reviewed by Bowers, 1991), our data suggest that increased ability to induce defenses at the juvenile stage is likely to increase plant resistance to generalist herbivores. Increased levels of iridoid glycosides, especially catalpol, usually deter generalist herbivores and/or decrease consumption rate (Bowers and Puttick, 1989; Biere et al., 2004; De Deyn et al., 2004; Wurst and van der Putten, 2007), potentially allowing induced juvenile plants to decrease subsequent damage by generalist herbivores. However, this increase in defenses also may enhance *P. lanceolata* susceptibility to specialist herbivores such as buckeye butterflies (Bowers, 1984; Pereyra and Bowers, 1988; Prudic et al., 2005; Talsma et al., 2008). Therefore, whether high induction capabilities at younger life stages in *P. lanceolata* is adaptive remains to be tested, but may strongly depend on the community structure of antagonist species present in the habitat. Alternatively, mature plant stages with already 16 times more constitutive defenses than juvenile stages may not benefit by investing in induced defenses. Thus, as seen in other plant species, age-dependent induced defenses in *P. lanceolata* may respond to variable cost and benefits of these defensive strategies as plants develop (Ohnmeiss and Baldwin, 2000; Van Dam et al., 2001; Boege et al., 2007; Orians et al., 2010).

Plant responses to sequential damage over a growing season may depend on and/or interact with age-dependent induced responses to herbivory. Two alternative scenarios can be expected, one where age-dependent induced responses can prevail over plant responses to sequential damage or, the opposite, where herbivore-induced indirect interactions alter plant defenses beyond the expected age-dependent induced response. To our knowledge, we currently lack empirical data testing these opposite scenarios. On one hand, recent empirical studies have shown strong age-dependent induced responses to herbivory (Van Dam et al., 2001; Boege et al., 2007; Barton, 2008; Tucker and Avila-Sakar, 2010), but these studies usually lack a consideration of the role of multiple and successive damage events over the course of plant development. Alternatively, studies that assess the effects of previous history of damage on plant responses to herbivory have shown both that previous damage events can increase plant induced responses after subsequent damage (Ruuhola et al., 2007; Roitto et al., 2009), or decrease plant ability to mount a localized or systemic induced response following subsequent damage (van Zandt and Agrawal, 2004; Viswanathan et al., 2007; Poelman et al., 2008; Erb et al., 2011). Although these latter studies assess the role of history of damage over extended periods of time, where plant ontogeny may interact with herbivore-induced indirect interactions, conclusions on the independent or combined effects of these factors on plant induced responses are hard to draw. Most of these studies vary widely in terms of plant life form (i.e., annual or perennial), herbivore-mediated indirect interactions (i.e., conspecific or heterospecific interactions), and time elapsed between initial and subsequent damage (i.e., from 48 h to >5 yr); making it difficult to assess whether changes in plant defense phenotypes and/or subsequent herbivore communities are the product of immunological memory and/or priority effects, age-dependent induced responses, or a combination of both. Our results show, that a week following the second damage event, mature plants with diverse frequency and intensity of damage (i.e., CC, CH, HC, HH) all had similar levels of defenses. Thus, for this plant-herbivore system and considering potential conspecific interactions occurring over successive generations (i.e., 5 wk), plant age seems to influence plant allocation of resources to defense more than past history of damage.

Evaluating changes in plant induced responses within and between above- and below-ground tissues as a function of plant age or history of damage is essential to understand temporal variation in plant-herbivore interactions. Herbivore-induced defenses not only may change the palatability and nutritional quality of a tissue for future herbivores, but also may lead to altered phytochemical expression across multiple plant tissues (Ohgushi, 2005; Kaplan et al., 2008). However, in this study, *P. lanceolata*'s short-term responses to 50% above-ground tissue removal by a specialist caterpillar did not translate into significant changes in root biomass or defenses (as also found in Darrow and Bowers, 1999). In accordance with our data, a recent meta-analysis of plant induced defenses within and across tissues following leaf and root herbivory demonstrated that herbivore damage to above-ground tissues usually results in stronger induced responses in leaves than in roots (Kaplan et al., 2008). Thus, if these observed short-term responses hold true throughout the growing season, then we should expect stronger temporal changes in above-ground herbivore population and/or community structure following leaf damage (especially in the case of juvenile plants) than in below-ground communities. Nevertheless, it is important to emphasize that the patterns described here may be system-specific. Here, we only assessed *P. lanceolata* responses to variable timing and severity of damage by a single specialist leaf-chewing herbivore, larvae of the Buckeye butterfly. It is the case for *P. lanceolata* that induced defenses within and across tissues can vary considerably as a function of herbivore identity. For instance, Bowers and Stamp (1993) showed that *P. lanceolata* damaged by the specialist *J. coenia* induced higher levels of iridoid glycosides than plants damaged by the generalist *Spilosoma congrua* (Arctiidae). Similarly, Wurst and van der Putten (2007) demonstrated not only that induction of iridoid glycosides depends on herbivore identity but also that herbivore-induced indirect interactions can vary as a function of initial and subsequent above- and below-ground herbivore species. Therefore, the patterns reported here may differ for different herbivore species and/or a combination of multiple antagonists damaging above- and below-ground tissues over time. Hence, future studies that assess the ecological consequences of history of damage and plant age-dependent induction within and across tissues for a wide diversity of above- and below-ground antagonists as well as more plant developmental stages will shed light on the ubiquity of the patterns observed here.

In summary, these results suggest that plant ontogeny not only dictates important shifts in plant investment in constitutive defenses, but it can also influence the ability of a plant to respond to herbivory, making ontogenetic trajectories a key feature in mediating plant-herbivore interactions. In contrast, variable frequency and intensity of damage in mature plant stages did not affect plant investment in defenses within as well as between tissues. This suggests that plant-mediated herbivore interactions throughout a season may depend strongly on plant age during initial and subsequent damage. For example, age-dependent induced resistance may shift the relative abundance or performance of generalist and specialist herbivores following initial damage to juvenile stages but not mature plant stages. Thus, in *P. lanceolata* and perhaps other systems, it is possible that ontogenetic trajectories in plant

growth and defenses leading to strong age-dependent induced responses may prevail over herbivore-induced indirect interactions. Yet, because herbivore-induced indirect interactions also vary as a function of herbivore identity and the lag of the localized and/or systemic induced response over time (van Zandt and Agrawal, 2004; Muola et al., 2010), more studies that address the effect of sequential damage by multiple shoot and root herbivores on plant defense phenotype and subsequent herbivore community structure throughout plant development are needed.

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genetic control of plant secondary chemistry are of importance to plant breeders, researchers, and forest managers, few studies have focused on forest trees, and fewer yet have investigated the interaction of a biotic agent and environment on plant defense responses. However, Hanover (1966) studied the heritability of five terpenoids present in pine, and Yanchuk et al. (2007) quantified tree defensive responses to insect attack. The reason for the dearth of studies on the heritability of tree secondary compounds and the response to biotic agents likely is due to the need for spatially replicated field trials of trees with known pedigree structure, that are of an appropriate age for biotic challenges of interest.

Mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae), is an obligate herbivore that has coevolved with lodgepole pine, *Pinus contorta* Douglas ex Louden var. *latifolia* Engelm. ex S. Watson (Pinales: Pinaceae) (Raffa and Berryman, 1983). Two ophiostomoid blue stain fungi, including *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield, are associated with *D. ponderosae* (Safranyik and Carroll, 2006). Beetles transport the fungi to the tree by either surface contact (fungi on exoskeleton) or via mycangia (specialized invaginations on beetle exoskeleton) (Paine et al., 1997; Krokene and Solheim, 1998). The blue stain fungi present an often lethal challenge for the host tree (Krokene and Solheim, 1998). Blue stain fungi grow throughout a tree's parenchyma cells, act to plug tracheids, and block nutrient and water flow. Trees typically respond to the fungi by allocating terpenoids to the site in an attempt to stop the growth of the fungi (Krokene et al., 2000; Huber and Bohlmann, 2006). For example in Petri dish assays, γ-terpinene, α-pinene, and abietic and isopimaric acids inhibited germination and growth of Ophiostomatales (Klepzig et al., 1996; Kopper et al., 2005). However, when bark beetle populations and their corresponding inocula are high due to mass attack, the defense system of healthy live trees do not inhibit the germination and growth of Ophiostomatales (Raffa and Berryman, 1983), which contributes to the mortality of the tree. Several important interactions play a role in this phenomenon.

During outbreaks of *D. ponderosae*, not all trees in the landscape, regardless of vigor, age and/or size will be mass attacked and killed by beetles. There are trees that are not landed upon; are landed upon but are not attacked; or are attacked but unsuccessfully colonized (Safranyik et al., 2004). The reasons that certain trees survive major outbreaks generally are unknown; however, as reported in other *Dendroctonus* spp-pine interactions, the environment strongly affects overall tree vigor (Ayres and Lombardero, 2000; Lombardero et al., 2000). However, genetic characteristics of the tree, and their interaction with the environment, also strongly affect any expression of resistance mechanisms of loblolly (Strom et al., 2002) and lodgepole pine (Yanchuk et al., 2007)

The *D. ponderosae* outbreak in British Columbia *P. c. latifolia* forests, and the tree breeding progeny trials established in the past by the British Columbia Forest Service, provided a rare opportunity to study the complex interactions of the host tree genetics, the insect, and a symbiotic, pathogenic fungus under replicated conditions in the field. We used 45 half-sibling families at two experimental sites, to study the effect of genotype and environment on the terpenoid chemical responses of *P. c. latifolia* to *G. clavigera*. Understanding host defense mechanisms involved in successful and unsuccessful *D. ponderosae* attacks and colonization may help expand our knowledge of why some trees appear to be resistant or tolerant to *D. ponderosae* attack. Our objectives were: (1) to examine the phenotypic variation in constitutive and induced secondary chemistry among half-sibling families and variations within individual trees; and (2) to estimate heritabilities for aspects of *P. c. latifolia* secondary metabolite defense resin chemistry.

Methods and Materials

Study Area Progeny trials for lodgepole pine were established in 1986 (prior to our study) at three sites in north central British Columbia, Canada. The sites were located near Prince George (Long. W 121° 35′ 18″ Lat. N 53° 29′ 18″, UTM Zone 10 East 593630 North 5927586), Wells (Long. W 121° 27′ 57″ Lat. N 53° 15′ 30″, UTM Zone 10 East 602371 North 5902142), and Williams Lake (Long. W 121° 21′ 42″ Lat. N 52° 09′ 11″, UTM Zone 10 East 612087 North 5779327). Plots will be referred to as Indian Point Creek (Indian Point), Bowron Lake (Bowron), and Moffat Creek (Moffat), respectively. Each plot was set-up in a randomized complete block design. In each plot, trees were grid planted at one meter by one meter spacing, in rows of four trees per family, with eight replication blocks per site. Plots were systematically thinned to one half the stocking density 10 yr prior to this study. Half-sibling open-pollinated families were common to all three sites, with varying local control wild stand seed lots also included. The seeds for families were sourced from local and non-local provenances, ranging from the north (Lat. N 53° 58′) to south (Lat. N 50° 42′).

Prior work at Indian Point provided preliminary information on the range of resistance parameters (antixenosis, antibiosis, and tolerance) to colonization by *D. ponderosae* (Yanchuk et al., 2007). Data reported in Yanchuk et al. (2007) were used to select families for this research on heritability of host responses to *G. clavigera*. We selected families in which 0–75% of the individuals showed signs of attack by *D. ponderosae*.

In addition to information from Yanchuk et al. (2007), family selections at Bowron and Moffat were based on direct observations of abiotic and biotic damage to individual trees. Trees were not used in this study if they had signs or symptoms of *D. ponderosae*; pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae); western gall rust, *Endocronartium harknessii* Hirats (Uredinales: Cronartiaceae); comandra blister rust, *Cronartium comandrae* Peck (Uredinales: Cronartiaceae); atropellis canker, *Atropellis piniphila* (Weir) Lohman & Cash (Helotiales: Dermateaceae); and/or stalactiform blister rust, *Cronartium coleosporioides* J.C. Arthur (Uredinales: Cronartiaceae). Families with greater than 50% of individuals showing signs and/or symptoms of these pest and pathogen agents were not included in the study.

In total, forty-five families were selected for this study. The number of individuals per family ranged from 16 to 26 across the two sites, for a total of 482 trees at Bowron and 405 trees at Moffat. Trees in the study had diameters measured at 1.4 m from the base of the tree (DBH), ranging from 5.8 to 16.7 cm. The treatments described below were conducted on individual trees prior to the *D. ponderosae* flight period in July 2006.

Fungal Cultures and Challenge Inoculations The fungus, *G. clavigera*, was isolated from adult *D. ponderosae* collected from Indian Point in August 2005. Samples were cultured by using methods described by Raffa and Smalley (1995) and Wallin and Raffa (2001). Cultures were grown in Petri plates on potato dextrose agar at 22°C and 85% relative humidity for 14–20 d and were used as the source for hyphal tip transfer. After establishing a pure culture, the number of transfers were limited to <10 transfers per dish, and visual inspections were used to prevent possible mutations. To obtain ample inoculum for field inoculations, ~600 1.5 mm agar samples with hyphal tips present were transferred to agar plates.

In July 2006, trees were inoculated with *G. clavigera* on the east and west side of the main stem at a point 1.4 m from the base of the tree. A 10 mm diam cork borer was used to excise bark and phloem. Inoculum containing hyphal tip material was removed from the petri dishes with a 4 mm diam cork borer. The inoculum was placed onto the exposed xylem tissue. The bark patch was re-secured to prevent desiccation and to promote propagation of the fungus. Prior to inoculation, each tree was sampled for constitutive terpenoids by using a 10 mm diam cork borer to remove undamaged phloem tissue. Host response was measured at 7 (sampled on the east side of each tree) and 12 (sampled on the west side of each tree) days following the initial inoculation. Post-inoculation sampling time points will be referred to hereafter as d 7 and d 12. At d 7 and 12, bark was removed from around the inoculation site, and the length of necrotic lesion was measured on the xylem. A 10 mm diam cork borer was used to remove phloem sample from the necrotic lesion. Samples were transported to a UNBC laboratory on dry ice and then stored in a −80°C freezer until it was shipped (also on dry ice) to the British Columbia Forest Service laboratory in Victoria, British Columbia where they were stored in liquid nitrogen until they were processed for terpenoid analysis.

Extraction of Terpenoids Constitutive and induced terpenoids were quantified from all 887 study trees in July 2006. Terpenoid quantity and composition were analyzed by gas chromatography with flame ionization detection (GC-FID). Outer bark tissue was separated from phloem tissue. The phloem was chopped to increase sample surface area before being placed into a vial with 4 ml of 240.5 ppm pentadecane solution as an internal standard (Sigma-Aldrich, 99% purity, Oakville Ontario, Canada). Pentadecane served as the internal standard since it is not present in *P. c. latifolia* phloem resin and is easily separated from the naturally present terpenoids (Hampel et al., 2005). After 48 h in solution, each vial was inverted to mix contents and then allowed to settle for an additional 24 h. Extracts (0.5 ml) were removed by using an air pipettor and placed into a 2 ml auto-sampler vial for GC-FID analysis.

The gas chromatograph (Perkin Elmer Clarus 500) was calibrated for all terpenoid standards and pentadecane at the British Columbia Ministry of Forests, Forestry and Technical Services Section, Research Branch Laboratory, Victoria, British Columbia, Canada. Samples were injected and volatized immediately at 200°C. The carrier gas, helium, flowed at a rate of 0.91 ml/min, into a split flow valve, which delivered the sample into the column. Compounds were separated with INNOwax column (J&W, 25 m in length a 0.2 mm i.d., 0.4 μm film thickness). The initial column temperature was 60°C and increased by 3.0°C/min to 85°C, then increased by 8.0°C/min to 170°C. The final step increased the temperature by 20.0°C/min to 250°C where it was held constant for 7 min. Peak areas were used to quantify terpenoids where the ratio of the area of a terpenoid was compared with the area of the internal standard. Following extraction, phloem samples were oven-dried and weighed. Total terpenoids were corrected based on dry weight per gram of sampled tissue.

Statistical Analyses Differences in terpenoid concentrations were assessed by using ANOVA in SAS (SAS Institute, version 8.2, Cary, NC 1999–2001). Individual and total terpenoid levels were analyzed for all trees and within individual trees by using repeated-measures ANOVA (among days after inoculation) with a model incorporating sites, replications within site, families, and the interaction of family and site. Homogeneity of variance and normality were tested with residual plots. Data were transformed as

necessary [log(x + 1)] to meet assumptions of tests prior to analyses, however, data for Δ-2-carene could not be transformed to meet assumptions.

Heritabilities (h^2) of all compounds were estimated as $h^2 = V_A / V_P$; V_A representing the additive genetic variance and V_P the total phenotypic variation. V_A is the family variance (V_F) multiplied by four and V_P is the sum of error variance, family variance and the family by site interaction. Family variance was multiplied by four to estimate additive genetic variance because variance among families in half-sibling trials account for only one-quarter of the additive genetic variance (Falconer, 1981). All heritability values were calculated by using the variance components obtained with the PROC MIXED procedure in SAS, with an auto regressive model; i.e., repeated-measures within trees were accounted for by the auto-regressive covariance parameter estimates (here tree families represented by the family variance and environmental variance was the sum variances of tree families, replication within site, and the errors from random effects). Heritability of non-repeated host defense parameters were calculated in the same manner, but without the auto-regressive repeated measure. Standard errors for all heritability values were estimated by adding the statement COVTEST to the PROC MIXED procedure after the dataset was identified (Becker, 1975). COVTEST displays estimated standard errors for the variables. Although the families in the study were not truly random effects, they were chosen to represent a broad cross section of families in the test that had differential *D. ponderosae* attack rates. It is assumed that the bias was minimal for the attributes that were measured and adequately represent genetic variation in this population.

Results

Variation in Constitutive and Induced Secondary Chemistry within Half-Sibling Families Constitutive terpenoid levels were measured at day 0 and induced terpenoids were measured at seven and 12 days post-inoculation with *G. clavigera*. Twenty-six terpenoids were identified from the phloem extracts; borneol, bornyl acetate, camphene, camphor, Δ-2- and Δ-3-carene, α-caryophyllene, α-copaene, α-cubebene, p-cymene, α-humulene, limonene, linalool, myrcene, ocimene, α-phellandrene, β-phellandrene, α- and β-pinene, pulegone, sabinene, α- and γ-terpinene, terpineol, terpinolene, and α-thujone; however, we focused on the seven terpenoids that comprised 94.19 to 95.78% of the total amount of monoterpenoids in constitutive and induced phloem tissue, respectively (Table 1). In order of descending abundance for constitutive tissue, the seven monoterpenoids were β-phellandrene, β-pinene, Δ-3-carene, α-pinene, limonene,

Table 1 Mean, standard error, percentage, and percentage change of total monoterpenes in lodgepole pine, *Pinus contorta latifolia*, phloem tissue following challenge inoculation with *Grosmannia clavigera* in the phloem tissue of half-sibling families at two locations in British Columbia, Canada[a]

Day following inoculation																							
Terpenoid	Bowron										Moffat												
	0				7				12			0				7				12			
	x	se	%	% Δ	x	se	%	% Δ	x	se	%	x	se	%	% Δ	x	se	%	% Δ	x	se	%
β-Phellandrene	11557	326	63	183	32734	1095	60	−12	28914	859	60	12850	560	62	146	31656	1012	59	−3	30853	1257	58
β-Pinene	1752	82	10	211	5451	290	10	−7	5055	270	11	2001	141	10	178	5558	273	10	6	5882	370	11
Δ-3-Carene	1305	69	7	408	6621	303	12	−16	5548	275	12	1828	101	9	289	7105	308	13	2	7269	425	14
α-Pinene	953	38	5	211	2961	176	5	−12	2616	152	5	1167	79	6	156	2985	215	6	10	3280	277	6
Limonene	831	51	5	69	1403	61	3	−4	1346	58	3	768	48	4	77	1361	75	3	3	1405	77	3
Myrcene	493	14	3	146	1214	38	2	−9	1102	31	2	588	24	3	111	1239	40	2	0	1242	50	2
Terpinolene	344	15	2	229	1131	72	2	−14	978	62	2	384	17	2	193	1126	41	2	−8	1041	52	2
Total Monoterpenes	18297	477	100	196	54140	1664	100	−11	47988	1352	100	20793	832	100	156	53276	1580	100	0	53334	2116	100

[a] Percentage change measured as [%Δ = (day/previous day * 100) − 100]; data were taken at 0, 7, and 12 d following inoculation with *G. clavigera* at Bowron Lake and Moffat Creek, near Wells and Williams Lake, British Columbia, respectively

myrcene, and terpinolene (Table 1). In induced tissue, the order of decreasing abundance was β-phellandrene, Δ-3-carene, β-pinene, α-pinene, limonene, myrcene, and terpinolene (Table 1).

Although the relative proportions of the 26 terpenoids were impacted by the simulated *D. ponderosae* attack, β-phellandrene, although somewhat variable, remained the most abundant terpenoid extracted from the phloem (Table 1). Similar to the constitutive tissue, β-phellandrene also was the most abundant monoterpenoid extracted from induced tissue at both times. Larger changes occurred at day 7, relative to the changes at day 12.

For the analyses where all three measurement periods had terpenoid levels 'pooled', there were significant differences in terpenoid levels between the two sites for Δ-3-carene, terpinolene, borneol, bornyl acetate, camphor, linalool, α-terpinene, γ-terpinene, terpineol, α-thujone, α-cubebene, and α-humulene. Quantities of all terpenoids were significantly influenced ($P \leq 0.05$) by replicates within sites with the exception of β-pinene, Δ-2-carene, ocimene, and α-thujone (Table 2). Family significantly impacted quantities of all terpenoids except for Δ-2-carene and α-thujone. Family by site interaction was only significant for the quantity of α-cubebene.

Analysis of variance revealed that all terpenoids varied with time of induction and replications within site, except for borneol, bornyl acetate, Δ-2-carene, and α-thujone. The interaction of tree (i.e., the repeated measure within tree) and site significantly impacted the quantities of Δ-3-carene, bornyl acetate, camphene, camphor, p-cymene, linalool, pulegone, sabinene, α-terpinene, γ-terpinene, α-thujone, α-caryophyllene, and α-humulene (Table 3). The amounts of terpenoids significantly increased or decreased by tree and family for all chemicals except for borneol, camphor, Δ-2-carene, pulegone, terpineol, α-thujone, α-caryophyllene, and α-cubebene. There were significant interactions ($P \leq 0.05$) among tree, family, and site in terms of the amounts of camphene extracted from the phloem tissue. No other terpenoids showed significant variation for this interaction. Constitutive effects on days 7 and 12 post-inoculation significantly influenced the amount of all terpenoids, except for Δ-2-carene; therefore, each time point after inoculation was treated separately (Table 4a-c).

Analysis of variance of the seven most abundant constitutive terpenoids prior to inoculation indicates that site and replication within site had significant effects on the amount of myrcene and terpinolene present in phloem tissue (Table 4a), but Δ-3-carene varied for site only. Family significantly impacted all seven of the most abundant terpenoids and no statistically significant interaction of family and site occurred (Table 4a).

For terpenoids induced seven days post-inoculation, replication within site and site significantly impacted seven and two, respectively, of the seven most abundant terpenoids (Table 4b). Similar to the pattern of variation in constitutive phloem tissue, quantities of all seven terpenoids were significantly influenced by family, but not significantly influenced by the interaction of family and site (Tables 4b). On day 12 post-inoculation with *G. clavigera*, terpenoids were influenced by all four sources of variation (Table 4c). In comparison to constitutive (Table 4a) and seven days post-inoculation (Table 4b),

Table 2 Repeated measures analysis of variance for between subject effects for terpenoids measured from phloem tissue of families of 45 half-sibling lodgepole pines, *Pinus contorta latifolia*, inoculated with *Grosmannia clavigera* at two sites in British Columbia, Canada[a]

Source of variation	SITE		REP(SITE)		FAM		FAM*SITE	
df	1		14		44		44	
Variable	F	Pr > F	F	Pr > F	F	Pr > F	F	Pr > F
β-Phellandrene	0.01	0.91	4.5	<0.001	3.09	<0.001	0.85	0.74
β-Pinene	0.89	0.35	1.31	0.20	3.26	<0.001	1.32	0.08
Δ-3-Carene	18.95	<0.001	3.34	<0.001	5.05	<0.001	0.71	0.92
α-Pinene	0	0.95	3.81	<0.001	3.02	<0.001	1.15	0.24
Limonene	0.25	0.62	2.26	0.01	4.64	<0.001	1.29	0.10
Myrcene	2.54	0.11	4.39	<0.001	2.77	<0.001	1.05	0.38
Terpinolene	7.52	0.01	3.58	<0.001	2.99	<0.001	0.93	0.61
Total Terpenoids	0.21	0.65	6.07	<0.001	2.22	<0.001	1.12	0.28
Total Monoterpenes	0.23	0.63	6.08	<0.001	2.21	<0.001	1.12	0.28
Total Sesquiterpenes	33.98	<0.001	2.38	<0.01	2.98	<0.001	1.14	0.25

[a] Variables were the family of half-sibling *P. contorta latifolia* (FAM) and the location of either Bowron Lake or Moffat Creek, near Wells and Williams Lake, British Columbia, respectively (SITE); there were eight replications within a site [REP(SITE)] and the quantities of terpenoids (ppm/g) were measured 0, 7, and 12 d post inoculation with *G. clavigera*

Table 3 Repeated measures analysis of variance for within subject effects for terpenoids measured from phloem tissue of families of 45 half-sibling lodgepole pines, *Pinus contorta latifolia*, inoculated with *Grosmannia clavigera* at two sites in British Columbia, Canada[a]

Source of variation	TREE		TREE*SITE		TREE*REP(SITE)		TREE*FAM		TREE*FAM*SITE	
df	2		2		28		88		88	
Variable	F	Pr > F	F	Pr > F	F	Pr > F	F	Pr > F	F	Pr > F
β-Phellandrene	628.34	<0.001	0.41	0.66	2.62	<0.001	1.64	<0.001	1.06	0.33
β-Pinene	642.97	<0.001	0.85	0.43	2.77	<0.001	1.37	0.01	1.08	0.29
Δ-3-Carene	1027.64	<0.001	5.56	<0.01	3.4	<0.001	1.43	0.01	1.12	0.22
α-Pinene	607.46	<0.001	0.07	0.93	2.95	<0.001	1.57	<0.001	1.03	0.41
Limonene	325.88	<0.001	0.13	0.88	2.09	<0.001	1.41	0.01	1.11	0.24
Myrcene	454.11	<0.001	1.86	0.16	2.64	<0.001	1.59	<0.001	1.15	0.16
Terpinolene	610.07	<0.001	0.95	0.39	2.42	<0.001	1.55	<0.01	1.06	0.34
Total Terpenoids	759.71	<0.001	0.35	0.71	2.96	<0.001	1.57	<0.001	1.12	0.22
Total Monoterpenes	759.54	<0.001	0.36	0.70	2.95	<0.001	1.57	<0.001	1.12	0.21
Total Sesquiterpenes	52.55	<0.001	4.22	0.01	2.51	<0.001	1.57	<0.001	1.12	0.22

[a] Variables were the individual tree (TREE); the family of half-sibling *P. contorta latifolia* (FAM); and the location of either Bowron Lake or Moffat Creek, near Wells and Williams Lake, British Columbia, respectively (SITE); there were eight replications within a site [REP(SITE)] and the quantities of terpenoids (ppm/g) were measured 0, 7, and 12 d post inoculation with *G. clavigera*

quantities of all of the seven terpenoids were significantly different for family but again not for the interaction of family and site (Table 4c).

Variation Among Families Quantitative changes in terpenoid abundance that occurred during induction from day 0 to day 7 varied between Bowron and Moffat, but there were some general trends; i.e., all 7 of the most abundant terpenoids increased at both sites. As such, total monoterpenoids extracted from phloem tissue of study trees also increased at both sites (Table 1).

Quantitative changes in terpenoid abundance varied much more between sites during induction from day 7 to day 12 (Table 1). β-Phellandrene decreased at Bowron and Moffat. β-pinene also decreased in the phloem of trees at Bowron, but increased at Moffat. Δ-3-Carene decreased in phloem tissue at Bowron, but increased at Moffat. During this induction the amount of α-pinene, limonene, and myrcene decreased in study trees at Bowron, but increased at Moffat. Similar to β-phellandrene, terpinolene decreased at both sites. Total monoterpenoids decreased in samples taken at Bowron, but increased at Moffat.

During the first induction period (day 0 to 7), families had variable changes in quantities of terpenoids; however, there were similar trends among the seven terpenoids that make-up the majority of the total monoterpenoid profile. For example, all 45 families had higher amounts of β-phellandrene, β-pinene, Δ-3-carene, α-pinene, and terpinolene at day 7 compared to the constitutive profile. Mean percent of limonene increased from day 0 to day 7 in 41 of 45 families at Bowron, and similarly, mean proportion of limonene increased in 43 of 45 families at Moffat. Myrcene levels increased in every case but two; only one family at Bowron, and one at Moffat. Terpineol was the only monoterpenoid to decrease from day 0 to 7 at Bowron and Moffat (Ott, 2010). At Bowron and Moffat, 98 and 89% of families, respectively, showed decreases in the amount of terpineol in their phloem resin. During the same time period, 67% of families at Bowron and 89% at Moffat decreased in total sesquiterpenoids (Table 1).

Unlike the first induction period from day 0 to day 7, the second period from day 7 to day 12 showed fewer consistent changes among families. This was not too surprising, considering the much smaller % change that occurred between day 7 and 12, vs. day 0 and 7 (Table 1). For example, twenty-nine percent of families and 42% of families increased in mean percent of β-phellandrene at Bowron and Moffat, respectively. Thirty-three and 51% of families at Bowron and Moffat, respectively, increased in mean percent of β-pinene and α-pinene. Levels of Δ-3-carene increased in 27% of families at Bowron and 40% of families at Moffat. Limonene increased in 51% of families at Bowron and 56% of families at Moffat. Myrcene increased in 29% and 44% of families at Bowron and Moffat, correspondingly. Mean amounts of terpinolene increased in 38% of families at both sites. Showing a trend contrary to the majority of monoterpenoids, terpineol increased in 98% of families at both sites. Total sesquiterpenoids increased in 80% of families at Bowron and 91% at Moffat from day 7 to 12.

Table 4 Analysis of variance by day after inoculation for quantity of terpenoids measured from phloem tissue of families of 45 half-sibling lodgepole pines, *Pinus contorta latifolia*, inoculated with *Grosmannia clavigera* at two sites in British Columbia, Canada[a]

Source of variation	SITE		REP(SITE)		FAM		FAM*SITE	
df	1		14		44		44	
Terpenoid	F	Pr > F	F	Pr > F	F	Pr > F	F	Pr > F
a				Day following inoculation 0				
β-Phellandrene	0.21	0.65	1.63	0.07	2.64	<0.001	0.98	0.51
β-Pinene	0.70	0.40	0.59	0.87	2.51	<0.001	1.38	0.06
Δ-3-Carene	25.31	<0.001	1.20	0.27	3.47	<0.001	0.86	0.73
α-Pinene	0.04	0.84	1.52	0.10	2.41	<0.001	1.11	0.29
Limonene	0.01	0.92	1.39	0.15	3.27	<0.001	1.19	0.19
Myrcene	3.96	0.05	1.83	0.03	2.18	<0.001	1.10	0.30
Terpinolene	5.11	0.02	1.70	0.05	2.49	<0.001	0.90	0.65
Total Terpenoids	0.62	0.43	1.59	0.08	2.17	<0.001	1.25	0.13
Total Monoterpenes	0.66	0.42	1.59	0.08	2.16	<0.001	1.25	0.13
Total Sesquiterpenes	7.16	0.01	1.24	0.24	2.34	<0.001	1.16	0.23
b				Day following inoculation 7				
β-Phellandrene	0.13	0.72	2.47	<0.01	2.78	<0.001	1.10	0.30
β-Pinene	2.10	0.15	1.54	0.09	3.07	<0.001	1.30	0.10
Δ-3-Carene	6.51	0.01	2.79	<0.001	3.42	<0.001	0.90	0.66
α-Pinene	0.02	0.88	3.58	<0.001	2.49	<0.001	1.24	0.14
Limonene	0.68	0.41	2.11	0.01	3.53	<0.001	1.33	0.08
Myrcene	0.40	0.53	2.56	<0.01	2.38	<0.001	1.24	0.14
Terpinolene	6.49	0.01	2.12	0.01	2.34	<0.001	1.23	0.15
Total Terpenoids	0.14	0.71	3.09	<0.001	2.04	<0.001	1.22	0.16
Total Monoterpenes	0.15	0.70	3.08	<0.001	2.04	<0.001	1.22	0.16
Total Sesquiterpenes	44.95	<0.001	3.84	<0.001	1.78	<0.01	0.94	0.58
c				Day following inoculation 12				
β-Phellandrene	0.32	0.57	6.23	<0.001	1.60	0.01	0.82	0.79
β-Pinene	0.01	0.92	3.25	<0.001	2.47	<0.001	1.05	0.38
Δ-3-Carene	4.88	0.03	6.64	<0.001	3.77	<0.001	0.89	0.67
α-Pinene	0.04	0.84	5.45	<0.001	2.11	<0.001	0.95	0.57
Limonene	0.12	0.72	3.73	<0.001	3.03	<0.001	1.16	0.23
Myrcene	0.01	0.94	7.06	<0.001	1.59	0.01	1.02	0.44
Terpinolene	1.39	0.24	5.98	<0.001	1.93	<0.001	0.94	0.58
Total Terpenoids	0.08	0.78	8.08	<0.001	1.30	0.09	0.89	0.67
Total Monoterpenes	0.08	0.78	8.05	<0.001	1.30	0.10	0.89	0.68
Total Sesquiterpenes	7.95	<0.01	2.98	<0.001	2.40	<0.001	1.27	0.12

[a] Terpenoid quantities (ppm/g) were measured 0, 7, and 12 d post inoculation with *G. clavigera*; variables were the location of either Bowron Lake or Moffat Creek, near Wells and Williams Lake, British Columbia, respectively (SITE); the family of half-sibling *P. contorta latifolia* (FAM) and there were eight replications within a site [REP(SITE)]

Heritability Estimates of Secondary Chemistry Analysis of *P. c. latifolia* host defense traits revealed that most measured traits responded to simulated *D. ponderosae* attack, and that the majority of these traits possessed significant additive genetic variation (Table 5). There was a large range of h^2 values estimated for *P. c. latifolia* primary and secondary defensive responses to simulated *D. ponderosae* attack (Table 5).

Δ-3-Carene levels had a heritability estimated at $h^2=0.48$ (s.e.=0.12), which was the highest estimated heritability value of all terpenoids (Table 5). Limonene, β-pinene, and terpinolene levels also had heritability estimates above 0.25 (Table 5). Of the seven most abundant terpenoids, α-pinene, β-phellandrene, and myrcene levels all had low heritability values compared to the others (Table 5). Total terpenoids, monoterpenoids, and sesquiterpenoids all had heritabilities

Table 5 Estimates of heritability (h^2) over time for the seven most abundant monoterpenes, total terpenoids, and the ratios between selected monoterpenes from phloem tissue of families of 45 half-sibling lodgepole pines, *Pinus contorta latifolia*, inoculated with *Grosmannia clavigera* at two sites in British Columbia, Canada[a]

Variable	h^2	s.e.
β-Phellandrene	0.20	0.06
β-Pinene	0.37	0.11
Δ-3-Carene	0.48	0.12
α-Pinene	0.22	0.07
Limonene	0.44	0.11
Myrcene	0.16	0.05
Terpinolene	0.29	0.08
Total Terpenoids	0.12	0.04
Total Monoterpenes	0.12	0.04
Total Sesquiterpenes	0.19	0.06
β-Pinene/Δ-3-Carene	0.58	0.15
Terpinolene/Myrcene	0.39	0.11

[a] Terpenoid quantities (ppm/g) were measured from *P. contorta latifolia* families at either Bowron Lake or Moffat Creek, near Wells and Williams Lake, British Columbia, respectively; estimates were based on covariance parameter estimates from tree families, replications within sites, and the errors (Falconer, 1981)

estimated to be lower than $h^2=0.25$. The estimated heritability of the ratio of β-pinene to Δ-3-carene was $h^2=0.58$ (s.e.=0.15). The estimated heritability of the ratio of terpinolene to myrcene was $h^2=0.39$ (s.e.=0.11). None of the sesquiterpenoids had heritability values estimated to be greater than 0.25.

Discussion

This study examined the genetic and environmental variation of tree chemical defenses against *D. ponderosae*, an insect that invades pine subcortical tissue, and one of its symbiotic fungi. We quantified changes in terpenoid abundance following simulated *D. ponderosae* attack and fungal invasion of subcortical tissue. Variations in defense parameters observed at different times following inoculation suggest a change in the allocation of carbon-based resources due to simulated attack by *D. ponderosae*. Constitutive and induced terpenoid levels were significantly affected by family, and low to medium heritabilities are involved in the expression of these responses.

Wounding and inoculating trees with *G. clavigera* resulted in necrotic lesions in phloem and xylem tissue. The formation of necrotic lesions following simulated bark beetle attack has been shown in many other studies and systems (Cook and Hain, 1986; Raffa and Smalley, 1988; Klepzig et al., 1996). Wounding and inoculation with *G. clavigera* significantly affected the abundance and quantity of terpenoids within the necrotic lesions at day 7 and day 12 post-inoculation. The levels of induction agree with those reported in Klepzig et al. (1996) and Raffa and Smalley (1988), i.e., the quantity of total terpenoids was higher in induced tissue than in constitutive tissue. The seven monoterpenoids that made up the major fraction of the total terpenoids in the tissue showed similar trends in how they changed over time, from day 0 to day 7 and day 7 to day 12 (Table 1).

The overall trend for the seven most abundant terpenoids was for them to increase by day 7 following inoculation. Monoterpenoid abundances were positively related as induction occurred from day 0 to day 7. However, this trend did not remain by day 12 post-inoculation. By day 12, most monoterpenoids at Bowron and Moffat had decreased or remained relatively stable. While it appears in the short-term that an attack by *D. ponderosae* induces and increases tree monoterpenoids appropriate for defense, this may indicate an immediate increase in defense related to the *D. ponderosae* threat, followed by a leveling off or even a return to the pre-induced state after the rapid response. This reduced level of response after day 7 also may be due to the limited challenge that we presented to the trees, i.e., most *D. ponderosae* attacks comprise many more sites of inoculation than did our experimental treatments.

Mass attack by *D. ponderosae* usually occurs within a short period of time following the onset of successful beetle colonization. This suggests that once an initial threat has been eliminated the tree may return to normal constitutive defensive levels and its regular functions. Somewhat contrary to this hypothesis, Klepzig et al. (2005) reported the amount of resin-based defenses of loblolly pines, *Pinus taeda* L., to be induced 105 days after simulated attack by southern pine beetle, *Dendroctonus frontalis* Zimmermann. However, both studies support the initial increase and trade-offs for the induction of defenses.

Differential expression of certain genes may regulate terpenoid expression due to herbivory or particular stress. For example, the mevalonic acid pathway (MVA) may be shut down or have isopentenyl diphosphate (IDP) (a building block of terpenoids) shunted away to promote the non-mevalonate pathway (MEP). This is due to the MVA producing primarily sesquiterpenoids, whereas the MEP produces primarily monoterpenoids, especially monoterpenoids of interest such as Δ-3-carene, myrcene, and α-pinene (Huber et al., 2004). All three of these particular monoterpenoids as well as others increased with induction by fungal inoculation. The hypothesis of trade-offs for induction of defenses may be further supported by sesquiterpenoids not being associated with *P. c. latifolia* defenses against *D. ponderosae* because sesquiterpenoids decreased due to simulated *D. ponderosae* attack.

The seven most abundant monoterpenoids that increase with induction have been shown to provide defense against bark beetles and other coniferous herbivores (Lindgren et al., 1996; Rocchini et al., 2000) or to affect pheromone-mediated aggregation and anti-aggregation (Pureswaran and Borden, 2005). In particular, Δ-3-carene is toxic to many conifer-invading insects (Cook and Hain, 1988; Rocchini et al., 2000; Fäldt et al., 2003; Carlow et al., 2006; D. W. Ross, Oregon State University, personal correspondence). In comparison to other terpenoids, Δ-3-carene occurs in relatively high concentrations in *P. c. latifolia*. This is also the case for β-pinene. However, the ranking of abundance of these two monoterpenoids changes with the onset of induction. Following inoculation, Δ-3-carene increased to a higher level than β-pinene. The change in proportion of these two terpenoids from the less toxic β-pinene to the more toxic Δ-3-carene strongly suggests that higher ratios of Δ-3-carene to β-pinene may provide a greater level of defense against *D. ponderosae*.

Myrcene has been shown to be important in the behavioral activity or synthesis of aggregation and anti-aggregation pheromones produced by *D. ponderosae* (Borden, 1982; Raffa and Berryman, 1983; Safranyik and Carroll, 2006). Raffa and Berryman (1983) reported that levels of terpenoids active in the aggregation response increase along with toxic terpenoids. This is an unintentional by-product of induction that *D. ponderosae* has adapted to and has used to its advantage. Myrcene has been shown, in conjunction with α-pinene, to be used to enhance the response of *D. ponderosae* to potential hosts and traps (Borden, 1982; Raffa, 2001; Pureswaran and Borden, 2005). Seybold et al. (2004) reported that in the Rocky Mountains, specific populations of *D. ponderosae* respond at higher rates when the aggregation attractant contains terpinolene vs. myrcene. Borden and colleagues tested the relative effects of these monoterpenes in British Columbia under laboratory (Borden and Pureswaran, 2006) and field (Borden et al., 2008) conditions. In the field, there was a synergistic effect of combining terpinolene with myrcene as part of the aggregation attractant for *D. ponderosae*. In our study, myrcene and terpinolene were induced at both sites. Terpinolene increased ~200% from the constitutive level, whereas myrcene increased by ~110-150%.

One challenge in estimating genetic variation in pest resistances is that there must be sufficient challenges by the pest to assess variation in the resistance traits of interest. Therefore, we simulated the challenge posed by the pest to assess complex interactions of the host tree genetics under replicated conditions in the field. The results support previous findings that there is large variation within and among trees for expression of these chemicals, but this variation also is composed of significant additive genetic effects in the population. In order to describe genetic variation of these defensive traits, it is important to offer a contextual framework of heritable traits provided by previous studies. Some studies categorize heritability values as high if the estimated value is ≥0.45 (King et al., 1997; Alfaro et al., 2000). Quencez and Bastien (2001) characterize moderate levels of heritability as between h^2=0.30–0.54. Whereas Roberds et al. (2003) state moderate levels of heritability are h^2=0.44 to 0.59, Hamilton et al. (2001) stated that the degree of genetic control or heritability of secondary defenses varies widely among plant species, but that it is generally thought to be in the order of 0.3. Zangerl and Berenbaum (2004) reported heritabilities of primary and secondary metabolites in their study that range between 0.048 and 0.728, with an average of approximately 0.3, similar to results shown by Hamilton et al. (2001). Within the context of the other studies discussed, heritabilities in our study were considered low (ranging from 0 to 0.25), moderate (0.25 to 0.5), or high (>0.5).

Our findings suggest that the majority of terpenoid variation within and among trees can be most consistently explained by family variation, relative to the other main effects or interaction. It was rare for terpenoids to vary significantly by site, which indicates a strong genetic factor influencing the levels secondary metabolites in this system. For example, only α-cubebene at day 0, bornyl acetate at day 7, and pulegone at day 7 varied significantly by the interaction of site and family—none of which are major terpenoid constituents of *P. contorta latifolia* resin. Moreover, the majority of changes from day 0 to day 7 and day 12 were primarily influenced by family and rarely by site. These findings should have important ramifications when considering *D. ponderosae* dispersal and host selection dynamics.

Heritability estimates for the seven most abundant terpenoids show four of the seven to have moderate heritability values [β-pinene, Δ-3-carene, limonene, terpinolene], and the other three to have low heritability values [β-phellandrene, α-pinene, myrcene]. However, they were all significant in that heritability estimates were greater than their standard error estimates.

While individual terpenoids have significant heritabilities, the ratios of particular terpenoids may be important. The ratio of β-pinene to Δ-3-carene abundance changed between the two variables. The ratio over the sampling period for these two terpenoids had the highest estimated heritability value at 0.58 (s.e.=0.15). This higher level of specific terpenoids or ratios between terpenoids may provide some advantage to individual trees, for example, when discussing possible anti-aggregation effects. The ratio of terpinolene to myrcene had a moderate heritability at h^2=0.39 (s.e.=0.11). This supports the idea of terpinolene as an important variable for attack by *D. ponderosae*, resulting in the beetle colonizing trees with higher levels of terpinolene, or terpinolene acting synergisti-

cally with myrcene to elicit higher flight responses to the *D. ponderosae* pheromone components (Borden et al., 2008).

Defensive traits that comprise constitutive and induced terpenoids are based primarily on quantities of secondary chemicals. Here, we have shown not only that *P. c. latifolia* have significant genetic variation in constitutive bark terpenoids, but also that genetic variation is present for chemical changes in response to attack, or at the very least, challenge from the fungal associate with *D. ponderosae*. This may have arisen from a long history of *D. ponderosae*, or other insects and diseases, and a complex of various selective pressures that have maintained low to moderate levels of genetic variation in defenses chemicals. While we have been able to show that monoterpenoid abundances are under significant genetic control, it is important to test defense hypotheses by linking these traits with negative impacts on the attacking species and/or host mortality, and further work is underway to document these relationships in *P. c. latifolia*. Also, while some traits may provide adequate defense during endemic or localized outbreaks of *D. ponderosae*, the current *D. ponderosae* population in British Columbia has killed a large proportion of the mature *P. c. latifolia* across the landscape. Yet, even in the midst of massive die off, some trees have not been killed. The results from this study provide a framework under which some traits for bark beetle defense can be considered—as well as the selection of families with less mortality/attack, or individuals that survived. This will be important in the future management plans and strategies for *D. ponderosae* system resistance, resilience, and tolerance, especially in the context of climate change, genetic improvement, population shifts, and landscape wide epidemic outbreaks.

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new areas in northern Alberta (Carroll et al., 2006), MPB has spread across the lodgepole×jack pine hybrid zone into stands of pure jack pine (Cullingham et al., 2011). Studies show that beetles can reproduce and develop in jack pine (Cerezke, 1995), as do its fungal associates (Rice et al., 2007). Jack pine is the most abundant pine species in the boreal forest which potentially could lead to the spread of MPB across the boreal forest to eastern Canada.

Global climate change might allow MPB to expand its range into formerly unoccupied lodgepole pine habitat and also enable further eastward invasion of jack pine (Williams and Liebhold, 2002). Climate change is expected to produce longer and more frequent droughts in many regions of the world (Breshears et al., 2009) that may influence the ability of trees to defend themselves against invading insects and diseases (reviewed in Franceschi et al., 2005). For plant species that are conservative water users, prolonged droughts will decrease carbon dioxide uptake and associated photosynthetic carbon assimilation due to stomata closure. Reduced carbon gain can result in the depletion of carbohydrate reserves for biosynthesis of defensive compounds, particularly carbon-based compounds such as terpenoids that could make trees more susceptible to biotic stress factors, such as bark beetles (McDowell et al., 2008; Breshears et al., 2009). Water deficit is one of the climatic variables used in climate-suitability models for MPB populations; in these models, it reduces the resistance of lodgepole pine to attack and subsequent development and survival of the beetle (Safranyik et al., 2010).

Increasing temperature predicted under a global climate change scenario also could alter volatile emission by potential host trees, since monoterpene emission is temperature dependent (Kesselmeier and Staudt, 1999), and might influence host finding by MPB. Two mechanisms are proposed to explain host finding by pioneering MPB. Beetles could visually orient towards and randomly land on potential host trees and then use gustatory cues to assess the suitability of the host (Raffa and Berryman, 1982). Alternatively, recognition and direct flight towards a host could be based on orientation to volatile organic compounds (VOC) emitted from the host (Moeck and Simmons, 1991) in combination with visual cues. MPB is able to detect and avoid non-hosts, which supports the hypothesis of direct flight as a host finding mechanism (Huber et al., 2000). Both mechanisms might act together; differences in VOC emission from different host trees grown under different conditions and with different levels of beetle infestation may be an indication of host suitability to orienting beetles. Successful attack and colonization of the host may be further influenced by the chemical composition of the bark of the host tree (Faccoli et al., 2005; Raffa et al., 2005).

Once a suitable host is found by a female pioneer beetle, α-pinene-one of the most abundant host monoterpenes in most pine species examined to date-is hydroxylated into the major aggregation pheromone *trans*-verbenol that attracts both sexes of MPB to initiate a mass attack (Pitman et al., 1968; Blomquist et al., 2010). As the colonization progresses, arriving males produce *exo*-brevicomin that attracts additional females until the optimum attack density is achieved, at which point both male and female beetles emit anti-aggregation pheromones that prevent further recruitment to the host tree (Rudinsky et al., 1974; Ryker and Libbey, 1982).

At least two MPB associated blue stain fungi, *Grosmannia clavigera* and *Ophiostoma montium*, also deplete tree defenses and kill their host (Reid et al., 1967; Rice et al., 2007) during the host colonization process. *Grosmannia clavigera* is more virulent than *O. montium* (Yamaoka et al., 1990) and often is used experimentally to stimulate tree defenses (Reid et al., 1967; Lieutier et al., 2009).

Trees that are mass attacked by the bark beetle plus associated fungi are defended with resin, a mixture of monoterpenes, sesquiterpenes, diterpenoid resin acids, and phenolic compounds characteristic of conifers (Keeling and Bohlmann, 2006) that act as physical and chemical defenses. Resin monoterpene composition differs between species, and the composition of individual trees in various pine species correlates with resistance to bark beetle attack (Sturgeon, 1979; Gollob, 1980).

In order to test the hypothesis that tree responses differ between lodgepole and jack pine trees, and also are affected by environmental conditions, we conducted a controlled environment experiment on seedlings subjected to water deficit (simulating drought conditions) and used the MPB fungal associate *Grosmannia clavigera* in conjunction with wounding to stimulate tree defenses.

The objectives were to: (1) develop a chemical profile of volatiles released from the two different host tree species; (2) evaluate if volatile chemical profiles vary within and between tree species when subjected to different environmental (water deficit vs. well watered) and biological (fungal inoculation, mechanical wounding, and control) treatments; and (3) determine if the monoterpenoid content of tissues is affected by the treatments and differs between tree species.

Methods and Materials

Seedling Source and Treatments Seedlings in their second growth cycle were used for all experiments. Lodgepole pine (*Pinus contorta* var. *latifolia* Engelm. ex Wats.) originated from west-central Alberta, while jack pine was from provenances in north-central Saskatchewan. Seedlings were grown through their first growth cycle under near-identical conditions at PRT Vernon, then packaged and placed in cold storage for approximately 3 month to satisfy dormancy requirements.

Dormant seedlings were shipped overnight to Edmonton, where $N=84$ seedlings per species were planted (pot size: 15×18 cm) in Sunshine Mix #4 (Sungro, Vancouver BC Canada) and maintained throughout the experiment in a walk-in growth chamber, with 16 L/8 h D, 200 μmol m^{-2} full spectrum light intensity, 20°C constant temperature and approximately 50% humidity. Fertilizer was applied with irrigation weekly (0.5 g/l 20 N -20 P -20 K), with additional watering provided as necessary until 1 week before the start of the experiment at which point both species had completed shoot elongation with an average height of 21.5 cm (±2.9 S.D) for lodgepole pine and 32.5 cm (±4.0 S.D) for jack pine seedlings.

Once terminal bud formation had been initiated, seedlings of each species were randomly divided into two groups and received one of two water regimes: water deficit or well watered. Seedlings in the water deficit group received 50 ml of water and those in the other group received 400 ml twice a week for the duration of the 6-wk experiment. In order to ensure that the water deficit seedlings were experiencing mild water stress conditions at the onset of the 6-wk experiment, water was withheld from these seedlings prior to the application of the fungal treatments. Seedlings in both water treatment groups were assigned randomly to one of three treatments: (1) mechanical wounding plus inoculation with *G. clavigera*; (2) mechanical wounding alone; and (3) untreated control. The aim of the first treatment was to stimulate tree defenses with the MPB fungal associate, and the last two treatments were positive and negative control treatments, respectively. Seedlings were inoculated with 5 μl of a *G. clavigera* spore suspension injected into a pouch made with a 23G1 PrecisionGlide™ needle. The spore suspension was prepared by using a sterile inoculation loop and transferring two loopfuls (20 μl) of spores of *G. clavigera* from a well sporulating strain collected from Fox Creek Alberta (54°24'N, 116°48'W) into sterile saline. Seedlings were inoculated at three points, equally distributed along the whole length of the stem. Mechanical wounding was applied in a similar manner but without the fungal inocula (Fig. 1).

For each tree species and water x biological treatment combination, four seedlings were randomly selected (total of 24 seedlings per species) and monitored for volatile emission, photosynthetic rate, stomatal conductance, and soil water content the day before inoculation ($t=0$), and at five time points post inoculation ($t=1$ day, $t=1$ week, $t=2$ week, $t=4$ week, and $t=6$ week). Twelve of the remaining seedlings of each treatment combination per species were destructively harvested at each time point ($N=2$) for chemical analysis of the bark (primarily constituting phloem tissue) of the current year and previous growth of the stem (referred to as current year and previous year bark) and needles. After 6 week, we harvested the seedlings that were used for acquiring volatile emission and physiology data for tissue analyses (below).

Soil Water Content and Gas Exchange Parameters Soil water content was measured using time domain reflectrometry (TDR) (Hillel, 1998). We measured the apparent dielectric constant of the soil with a Tektronix 1502B (Beaverton, OR, USA) and used the empirical equation for organic soils in Robinson et al. (2003) to relate it to water content. Rate of photosynthesis and stomatal conductance were measured using a LI-6400 (LI-COR Biosciences, Lincoln, NE, USA). The instrument settings were: leaf area 1.5 cm^2, flow 300 μmol/sec, and Quantum flux lamp at 200 μmol/m^2sec (equivalent to light intensity in growth chamber).

Volatile Collection Volatile organic compounds (VOCs) released from seedlings were collected 1 day after the physiological data were recorded from the same plant. An oven bag (LOOK®, 45×55 cm) was imposed over the

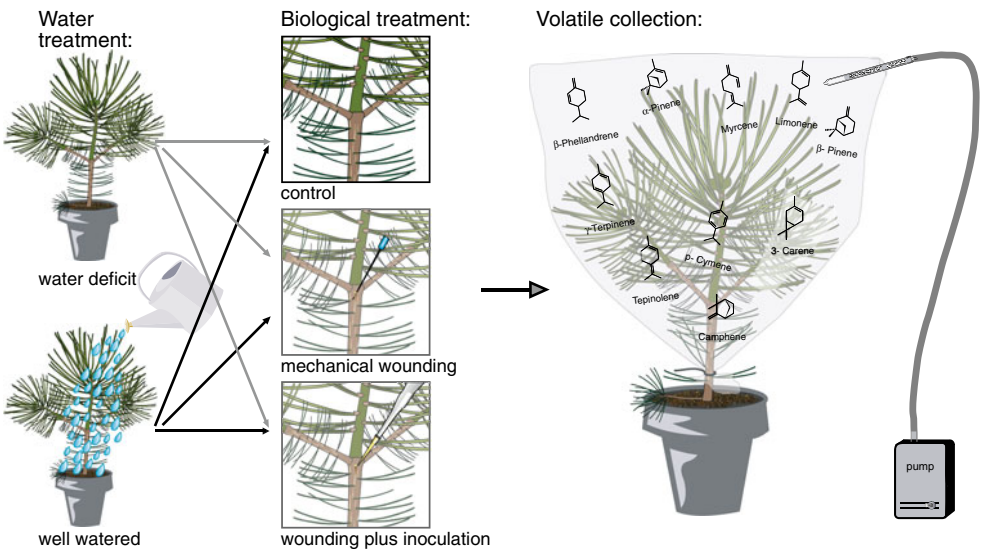


Fig. 1 Methodology scheme showing the two different water treatments: water deficit and well watered, the three biological treatments: control, mechanical wounding and wounding plus inoculation with *Grosmannia clavigera*, and collection of VOCs

whole seedling and closed near the base of the stem with a cable tie. An absorbent tube [Porapak Q (OD 6 mm, length 110 mm; absorbent: front layer 150 mg, back up layer 75 mg; separated by glass wool) SKC Inc., PA, USA] was inserted into the bag at the top of the seedling and affixed with Parafilm®. A small hole was placed on the other side of the oven bag to maintain constant air pressure inside the bag. Volatile emissions were collected for 4 h at a constant flow rate of 200 ml/min. After collection, the sorbent tubes were capped and stored at −40°C until extraction. Air samples were collected at two locations of the growth chamber to control for possibility of contamination with chamber air.

Porapak Q tubes were extracted with 1 ml of dichloromethane (Sigma-Aldrich, St. Louis, MO, USA) spiked with 0.01% (v/v) tridecane (Sigma-Aldrich, St. Louis, MO, USA) as surrogate standard and subsequently stored at −40°C before GC analysis.

Tissue Extracts Seedlings were harvested outside of the chamber in which VOCs were collected. During seedling harvest, bark and needle samples were frozen rapidly in liquid nitrogen, and stored at −40°C prior to extraction. Tissue was ground in liquid nitrogen, and 100 mg of the tissue were transferred to a 1.5 ml microcentrifuge tube. Samples were extracted twice with 0.5 ml dichloromethane and 0.01% tridecane as surrogate standard. After adding solvent, samples were vortexed for 30 sec, sonicated for 10 min, subsequently centrifuged at 13,200 rpm and 0°C for 15 min, and placed in a freezer for at least 2 h to let the pellet freeze. Extracts were transferred into an amber GC vial and stored at −40°C before GC analysis.

GC Analysis Samples (1 μl) were injected in an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) with an HP Innowax (Agilent Technologies) column (I.D. 0.32 mm, length 30 m), helium carrier gas flow at 1.8 ml/min, temperature 50°C for 2 min, increased to 160°C by 5°C per min, and then to 250°C by 20°C.

Peaks were identified using the following standards: Borneol, pulegone, α-terpinene, γ-terpinene, α-terpineol (Sigma-Aldrich, St. Louis, MO, USA), camphor, 3-carene, α-humulene, terpinolene, α-thujone and β-thujone, (-)-α-pinene, (-)-β-pinene, (S)-(-)-limonene, sabinene hydrate, myrcene, (-)-camphene, *p*-cymene (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate, *cis*-ocimene (SAFC Supply Solutions, St. Louis, MO, USA), β-phellandrene (Glidco Inc., Jacksonville, FL, USA).

Calibration with standards allowed for analysis of quantitative differences among samples of differently treated seedlings at different time points. For all samples, peaks were integrated and peak area was compared for qualitative and quantitative differences among samples of differently treated seedlings at different time points.

After all samples were analyzed with the GC, subsequent analysis with GC-mass spectrometry showed that our method was unable to separate myrcene and α-phellandrene. From a different experiment, we know that α-phellandrene hardly occurs in jack pine seedlings and makes up to 35% of the peak in lodgepole pine seedlings.

Statistical Analyses The VOC emission data and the seedling physiology data collected from the same trees were analyzed using the R statistical language (R development core team, 2010). In order to address how volatile emission of the seedlings changed due to the environmental and biological treatments and their associated change in physiology, we carried out a canonical redundancy analysis (RDA; Legendre and Legendre, 1998) using the rdaTest package (Legendre and Durand, 2010). RDA axes were tested for significance by permutations with the vegan package (Oksanen et al., 2010) also through the R statistical language. Explanatory variables included water and biological treatments, as well as soil water content, photosynthesis and stomatal conductance. Total and individual monoterpenes of all time points were the response variables. Due to technical difficulties during the extraction process, two time points (weeks 1 and 6) were discarded from the jack pine dataset. In order to evaluate whether the resulting lack of correlation between soil water content and physiological parameters in jack pine was due to the missing two time points, we also carried out an RDA for lodgepole pine with the same time points omitted, the results remained the same.

To assess the effect of environmental treatments on total monoterpene emission from both tree species over time, Mann–Whitney *U* tests were used. The effect of the biological treatments was assessed using repeated measures ANOVAs with subsequent pairwise comparison with Bonferroni correction on water deficit and well watered seedlings separately using SPSS Statistics 17.0. Soil water content and plant physiology results are presented using bar graphs with error bars showing 95% confidence intervals, non-overlapping bars represent a significant difference.

All tissue extract data were analyzed using SPSS Statistics 17.0. Total monoterpenes were analyzed using repeated measure ANOVA. Individual monoterpene data were transformed with log(x+1) to meet the assumptions of normality and analyzed for all tissues separately using ANOVA. In cases in which the data violated the assumptions of an ANOVA, non parametric tests were conducted on non transformed data. Interspecific differences were analyzed using *t*-test.

Results

Soil Water Content and Gas Exchange Parameters The water deficit seedlings were not watered the week before

the experiment started; therefore, there was a significant difference in soil water content between the well watered and water deficit seedlings on the first day of the experiment (Fig. 2). Through the duration of the experiment, the water content of the soil in the water deficit treatment decreased to almost 0%, whereas soil water content of well watered seedlings stayed constant between 20% and 30%.

Independent of the biological treatments, the photosynthesis rate in lodgepole pine decreased over time in the water deficit group, but not in the well watered group (Fig. 3a). In jack pine, neither the biological nor the water treatments had an effect on photosynthesis rate. Stomatal conductance was generally higher in lodgepole than jack pine but it decreased sooner due to water stress in lodgepole pine and reached 1/3 of the jack pine stomatal conductance by the end of the experiment (Fig. 3b).

Chemical Profiles The main differences in the chemical profile emitted by seedlings of the two pine species are that lodgepole pine (LP) emits a higher percentage of β-phellandrene (27%±2.25 SE) than jack pine (1%±0.26 SE) and jack pine (JP) releases more α-pinene (27%±3.25 SE) than lodgepole pine (7%±0.80 SE) (Fig. 4). 3-Carene makes up more of the volatile chemical profile in jack pine than lodgepole pine (21%±3.99 SE in JP, 7%±1.76 SE in LP).

Volatile Emission The first axis of the lodgepole pine redundancy analysis (RDA) ($P<0.001$) and of the jack pine RDA ($P=0.006$) was significant. RDA triplots (Fig. 5) illustrate the relationship between the gas exchange parameters and volatile emission of the seedlings and water stress and biological treatments for each tree species.

In both species, 3-carene emission correlated with fungal inoculation. Emission of the major compounds, β-phellandrene in lodgepole and α-pinene in jack pine correlated with the physiological state (photosynthesis and stomatal conductance) of the seedling. The physiological parameters of stomatal conductance and photosynthesis correlated positively with soil water content in lodgepole but not jack pine seedlings.

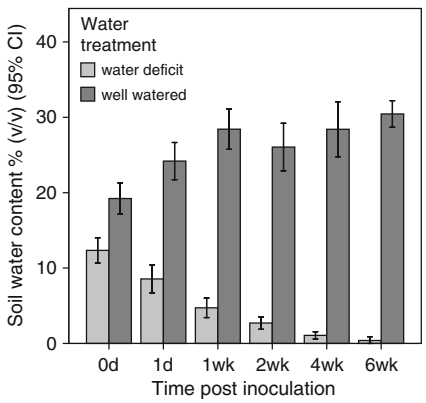


Fig. 2 Soil water content over time measured for pots containing lodgepole and jack pine seedlings. For each time point, data for the two species were combined. Error bars represent the 95% confidence interval. Bars with non overlapping error bars are significantly different from each other

In both lodgepole pine and jack pine, water deficit seedlings released significantly lesser amounts of total monoterpenes starting at week 4 of the experiment (Mann–Whitney U, lodgepole pine: $U=21$, $z=-2.94$, $P=0.003$; jack pine: $U=26$, $z=-2.66$, $P=0.008$) and thus, all further analyses were conducted on water deficit and well watered seedlings separately. Biological treatments had a significant effect on total monoterpene emission in water deficit lodgepole pine seedlings [repeated measures ANOVA, $F(2,8)=6.329$, $P=0.022$; Fig. 6]. Subsequent pairwise comparison with Bonferroni correction of the timepoints revealed that 1 day after biological treatment application greater amounts of VOCs were emitted from lodgepole pine than at any other timepoint ($P=0.006$; Fig. 6). Further, pairwise comparison of the biological treatments showed that lodgepole pine seedlings that were inoculated with *G. clavigera* emitted significantly greater amounts of monoterpenes than controls ($P=0.029$). Mechanically wounded seedlings also emitted greater amounts of volatiles than control seedlings but not at a significant level ($P=0.066$). Well watered lodgepole pine demonstrated the same trends but showed no significant differences. In jack pine, the biological treatments had no significant influence on VOC emission. Lodgepole pine seedlings emitted lesser amounts than jack pine but the difference was not significant.

Tissue Extracts The number of detectable monoterpenes in the tissue extracts of both species was higher than in the volatile profiles (Table 1). Sabinene hydrate, *cis*-ocimene, α-thujone, and pulgeone occurred only in lodgepole pine and were never detected in jack pine.

The overall needle monoterpene content in lodgepole pine seedlings is lower compared to previous year and current year growth bark [repeated measures ANOVA, $F(1.80,127.70)=118.37$, $P<0.001$; Fig. 7]. *cis*-Ocimene is the only terpenoid that did not follow this pattern and it occurred in higher concentration in the needles. All further analyses were conducted on the different tissues separately. The environmental treatment affected terpinolene, which occurred in higher concentrations in the current year and previous year bark of watered seedlings [ANOVA, $F(1,70)=3.999$, $P=0.028$ and $F(1,70)=5.059$, $P=0.049$, respectively] than in the water deficit seedlings. Less bornyl acetate occurred in the previous year's bark of watered seedlings [ANOVA, $F(1,70)=5.265$, $P=0.025$] compared to seedlings subjected to water deficit. The biological treatments had no effect on any of the measured terpenoids in the tissue extracts.

Like lodgepole pine, jack pine needles had a significantly lower overall monoterpene content than current year or previous year bark [repeated measures ANOVA, $F(2,142)=$

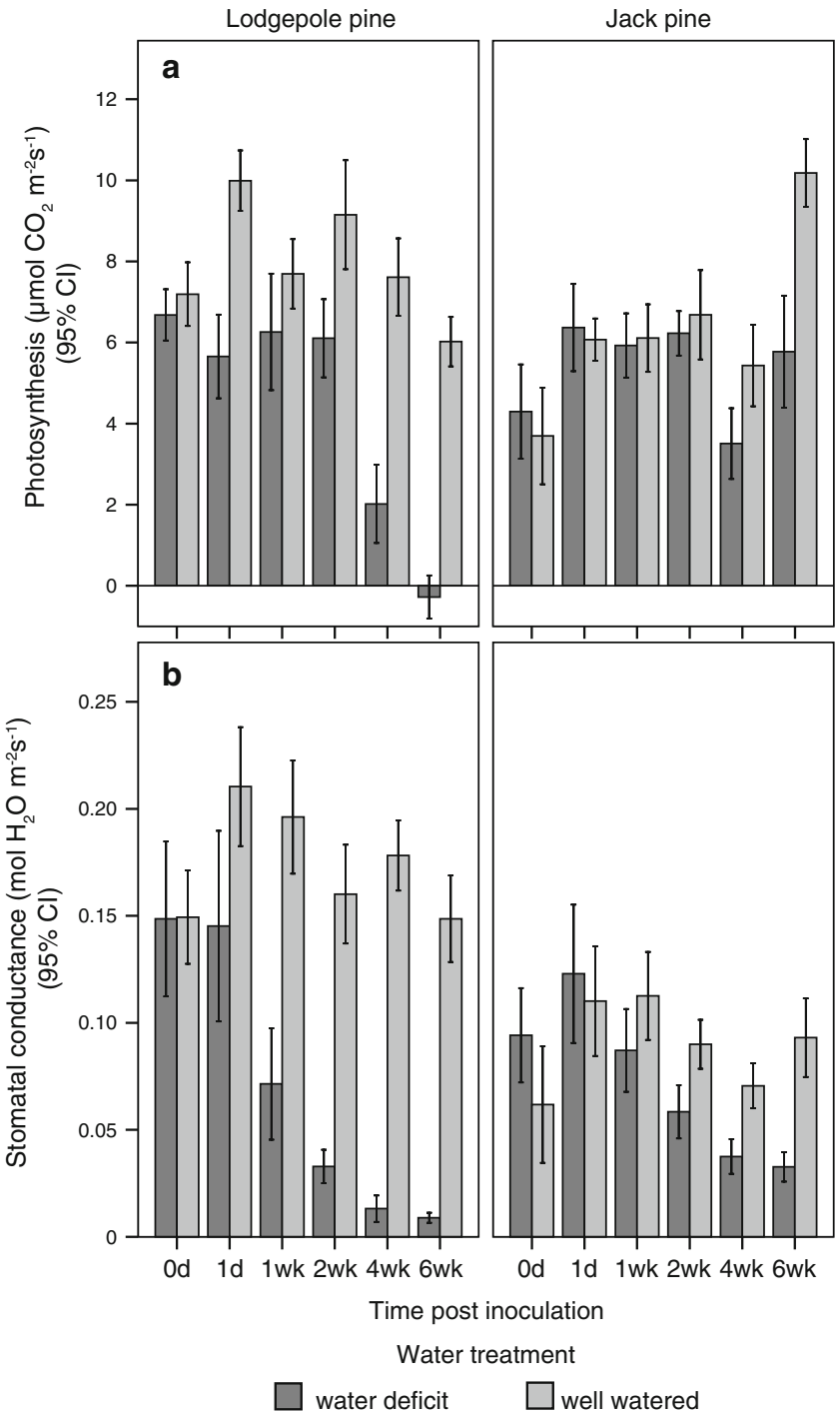


Fig. 3 Changes in gas exchange parameters over time shown for both water treatments in lodgepole and jack pine seedlings (**a**) rate of photosynthesis (**b**) stomatal conductance. Error bars represent the 95% confidence interval. Bars with non overlapping error bars are significantly different from each other

115.66, $P<0.001$; Fig. 7]. The environmental treatment had no effect on monoterepene content of jack pine tissues. The bornyl acetate content in the previous year bark of fungal-inoculated seedlings was higher than in wounded seedlings [Kruskal-Wallis, $H(2)=16.467$, $P<0.001$; Fig. 8].

The overall monoterpene content of all tissues increased in both species over time independent from any manipulation (Fig. 9). Both species exhibit similar terpenoid content in collected bark tissues, but jack pine needles contain more terpenoids compared to those from lodgepole pine [*t*-test, $t(117.664)=3.413$, $P=0.001$; Fig. 7].

Discussion

Water regime manipulation and stimulation of plant defenses affected the emission of VOCs differently in the two studied

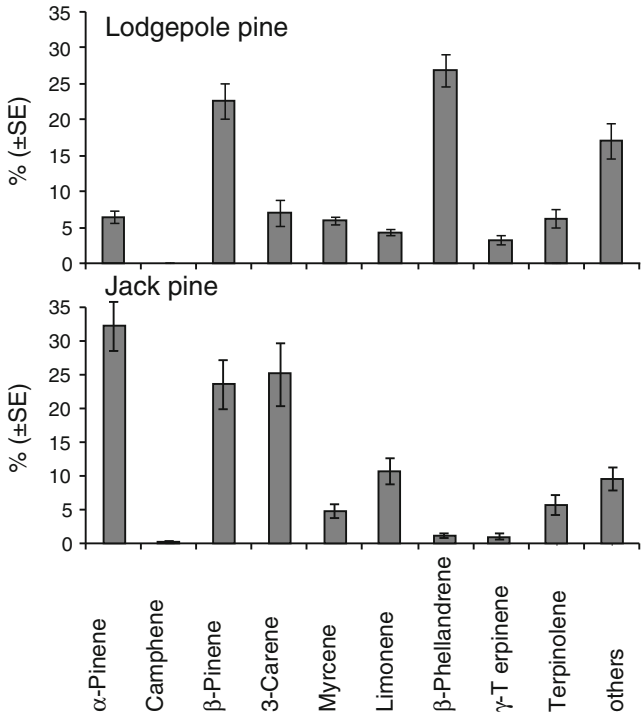


Fig. 4 Chemical profiles presented as percent of volatile organic compounds emitted from lodgepole pine and jack pine seedlings based on peak area in GC analysis. Error bars indicate standard errors

pine species. In general, jack pine emitted higher amounts of 3-carene (21%), compared to lodgepole pine (7%) while lodgepole pine released higher amounts of β-phellandrene (27%) than jack pine (1%) (Fig. 4). β-Phellandrene is the only monoterpene that attracts MPB in the absence of aggregation pheromones when released at high doses (Miller and Borden, 1990), which could mean that pure jack pine stands might fail to attract pioneer beetles flying actively into the region compared to lodgepole pine stands.

The chemical profile of jack pine seedlings tested in our study contained 21% of 3-carene on average, similar to previous reports of jack pine from central Saskatchewan, Canada (Pauly and von Rudolff, 1971) and consistent with the profile of mature jack pine in the Smoky Lake region of Alberta (unpublished data). Within the wide range of jack pine in North America, there are surprisingly few records available on chemical profiles of jack pine, and almost all records are from the most southern extension of the jack pine forest in Wisconsin, USA. In those studies, the chemical profiles consist primarily of α- and β-pinene; 3-carene was either not found or occurred only in trace amounts (Zavarin et al., 1969; Wallin and Raffa, 1999; Erbilgin et al., 2001; Aukema et al., 2010). An early study that directly compared resin monoterpene composition among lodgepole pine, jack pine, and their hybrids found 3-carene to be a signature compound in lodgepole pine that could distinguish it from jack pine (Zavarin et al., 1969). This large variability of 3-carene in jack pine monoterpene profiles suggests either that there are two or more phytochemical phenotypes in jack pine, similar to lodgepole pine (Forrest, 1980), or that there is a greater degree of introgression of lodgepole pine genes into jack pine in the western extent of the jack pine range than originally assumed. A similar variability of 3-carene content occurs in Scots pine seedlings from different provenances in Turkey and Finland (Semiz et al., 2007), suggesting that terpenoid synthesis is under genetic control and differs by geographical origin of the seeds. Clarification of the role, if any, of 3-carene in jack pine profiles is essential in order to predict the potential interaction between MPB and jack pine in the boreal forest.

The chemical profile measured from lodgepole pine seedlings in our study is in agreement with existing published profiles (Zavarin et al., 1969; Pauly and von Rudolff, 1971; Pureswaran et al., 2004a). The consistency in monoterpene profiles of lodgepole pine resin has enabled the use of chemosystematics across the geographic range of the species (Forrest, 1980). Chemical profiles are used to distinguish subspecies/varieties of lodgepole pine, which are: subsp. *contorta*, *bolanderi*, *murrayana*, and *latifolia* (Critchfield, 1957). Chemotyping only failed to distinguish the lodgepole pine subspecies *murrayana* and *latifolia* (Forrest, 1980; Bohm, 2009). Our results underscore the need for a thorough study to chemotype jack pine trees across its broad range.

Seedlings of both species emitted an overall lower quantity of VOCs under water deficit conditions, which was evident at day one of the experiment but significant at week four (Fig. 6). Although emission of VOCs is not thought to be controlled by stomatal conductance (Sharkey, 1991; Kesselmeier and Staudt, 1999; Blanch et al., 2007), in our study stomatal conductance may have indicated the general condition of seedlings and thus indirectly affected the efficiency of monoterpene synthesis in the plant to increase monoterpene emission rates. Similarly, VOC emission was strongly reduced by severe drought conditions and linked to stomatal closure in common Mediterranean woody species including *Pinus halepensis* (Llusià and Peñuelas, 1998). Although in a similar study, *Pinus halepensis* seedlings exposed to moderate drought conditions exhibited no change in VOC emission as a result of water deficit (Blanch et al., 2007). In our study, stomatal conductance explained the release of some of the most abundant monoterpenes in lodgepole pine and jack pine. Likewise, Niinemets et al. (2002) concluded that stomatal conductance in *Pinus pinea* affected the emission of alcohols, aldehydes, carboxylic acids, and oxygenated monoterpenoids. This finding should be considered in monoterpenoid emission models that currently use a simple equation that relies mainly on temperature to describe the monoterpene efflux from foliage (Guenther et al., 1991) and does not consider any role of

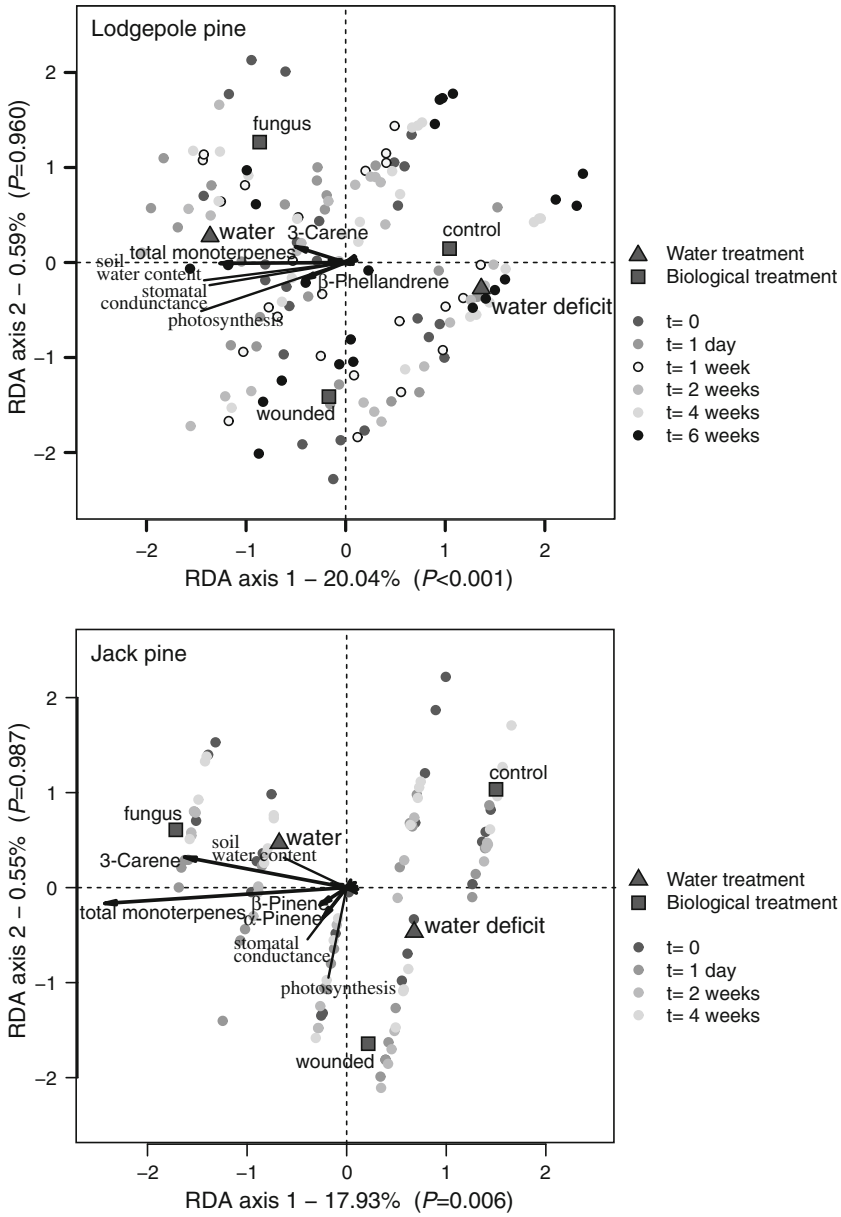


Fig. 5 Canonical redundancy analysis (RDA) triplots (scaling 2) illustrating the effect of environmental and biological treatments as well as seedling physiology on volatile emission of individual and total monoterpenes in lodgepole and jack pine

stomatal conductance in VOCs emission (Sharkey, 1991; Kesselmeier and Staudt, 1999).

Stomatal conductance and the resulting gas exchange that is essential for the carbon reactions of photosynthesis depend on the water transport from soil to leaf. Plant species categorized as isohydric exhibit tight control over stomatal aperture in response to soil water deficit as a means to regulate water loss; whereas species classified as anisohydric do not (Tardieu and Simonneau, 1998; McDowell et al., 2008). Stomatal conductance in lodgepole pine seedlings in the current study was higher than that in jack pine and responded more quickly to a decrease in soil water content (Fig. 3). Jack pine appears to be better adapted to low water availability than lodgepole pine, which is consistent with the success of jack pine in dry and nutrient poor soils across its natural range (Vidaković, 1991). In our study, soil moisture content influenced the physiological state of pine species differently. The RDA results (Fig. 5) support the findings that jack pine is better adapted to water deficit conditions, as the physiological state of lodgepole pine but not that of jack pine was correlated with soil water content.

Fungal inoculation of seedlings with an MPB associated fungus also altered the VOC emissions in both pine species. 3-Carene emission increased and was correlated with fungal inoculation in both lodgepole and jack pine (Fig. 5). Mature jack pine trees inoculated with the pine engraver, *Ips pini*, associated fungus *Ophiostoma ips* had increased 3-carene phloem content (Raffa and Smalley, 1995). Jost et al. (2008) inoculated mature lodgepole and jack pine trees as well as their hybrids with MPB associated fungi and collected bark

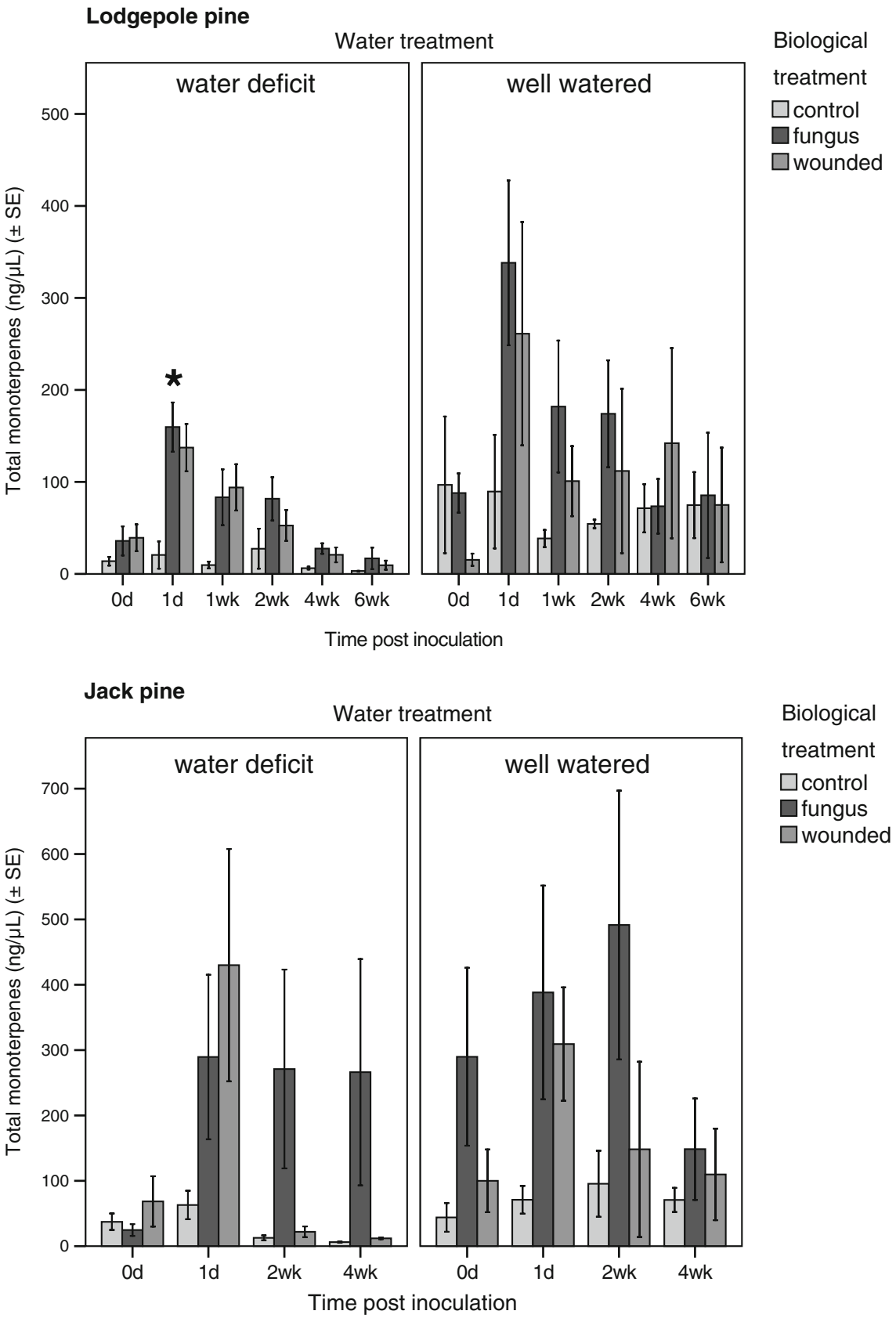


Fig. 6 Effect of water and biological treatment on total monoterpene emission by lodgepole and jack pine seedlings over time. Error bars indicate standard errors. The asterisk indicates the significant difference of total monoterpene emission in fungal inoculated water deficit lodgepole pines compared to the control treatment (P=0.029)

Table 1 Monoterpene content of the different tissues in lodgepole and jack pine (% of amount ng/mg tissue)

Monoterpene	*Lodgepole pine*						*Jack pine*					
	Needles mean (±SE)		Current year bark mean (±SE)		Previous year bark mean (±SE)		Needles mean (±SE)		Current year bark mean (±SE)		Previous year bark mean (±SE)	
α-Pinene	6.55	0.78	5.57	0.69	6.65	0.81	24.03	4.96	24.55	6.24	29.75	6.27
Camphene	3.19	1.42	2.47	0.95	3.87	1.26	0.91	0.44	0.97	0.31	1.95	0.33
β-Pinene	20.78	3.01	25.19	3.41	27.23	3.55	6.92	1.81	8.08	1.53	14.64	3.04
3-Carene	15.64	2.64	12.97	3.16	15.39	3.17	38.66	8.00	47.12	8.78	42.6	8.89
Myrcene	7.31	0.32	5.69	0.18	5.35	0.25	4.87	0.26	3.29	0.09	3.09	0.07
Limonene	3.94	0.22	8.14	1.72	6.43	0.85	19.12	5.52	12.77	4.04	4.84	1.28
β-Phellandrene	31.99	3.77	34.30	2.83	27.25	3.99	2.63	1.19	1.05	0.22	1.04	0.13
cis-Ocimene	1.68	0.15	0.46	0.17	0.34	0.10	0	0	0	0	0	0
γ-Terpinene	0.50	0.05	0.15	0.02	0.17	0.01	0.34	0.11	0.27	0.05	0.22	0.04
Terpinolene	0.13	0.07	0.36	0.07	0.37	0.06	0.66	0.15	1.03	0.22	0.82	0.2
α-Thujone	0.37	0.04	0.13	0.01	0.13	0.01	0	0	0	0	0	0
β-Thujone	0.61	0.06	0.22	0.02	0.22	0.02	0.22	0.12	0.09	0.06	0.07	0.04
Sabinene hydrate	0	0	0.01	0.01	0.02	0.01	0	0	0	0	0	0
Camphor	1.37	0.14	0.49	0.05	0.55	0.07	0.40	0.22	0.16	0.11	0.13	0.07
Linalool	0.34	0.13	0.15	0.03	0.17	0.05	0.07	0.07	0.05	0.03	0.03	0.03
Bornyl acetate	3.73	1.71	2.83	1.42	5.1	2.01	0.89	0.23	0.33	0.10	0.45	0.10
Pulgeone	0.76	0.08	0.29	0.03	0.29	0.02	0	0	0	0	0	0
α-Humulene	0.21	0.09	0.07	0.02	0.12	0.04	0.05	0.05	0	0	0	0
α-Terpineol	0.01	0.01	0.08	0.03	0.04	0.02	0.22	0.09	0.24	0.07	0.36	0.08
Borneol	0.88	0.20	0.42	0.20	0.31	0.09	0	0	0	0	0.02	0.02

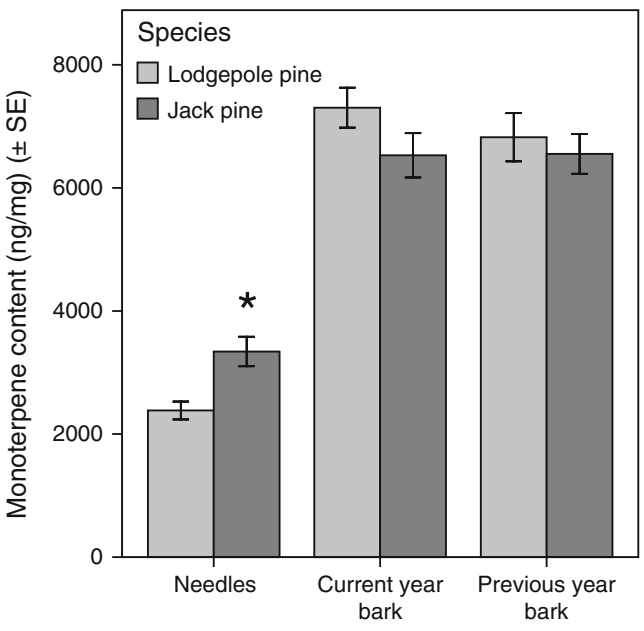


Fig. 7 Total monoterpene content in the three different tissues of lodgepole and jack pine seedlings. Error bars indicate standard error. The asterisk indicates the significant difference in monoterpene content between lodgepole and jack pine needles

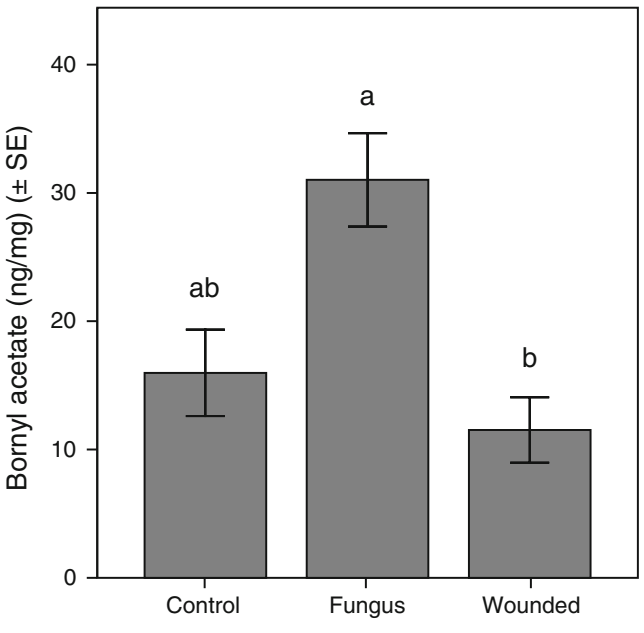


Fig. 8 Different bornyl acetate concentrations in previous year's bark of jack pine caused by the biological treatments. Kruskal-Wallis test was used followed up by Mann–Whitney *U* tests. A Bonferroni correction was applied and so all effects are reported at a 0.025 level of significance. Different lowercase letters indicate a statistically significant difference

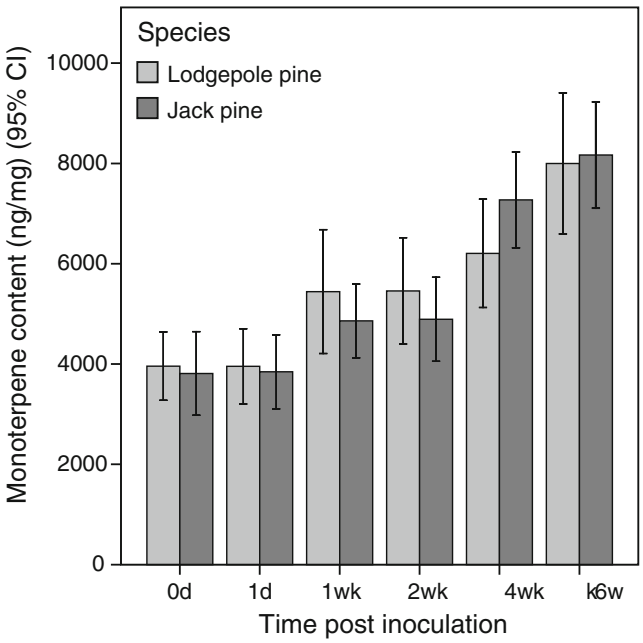


Fig. 9 Total monoterpene increase over time in all tissues of lodgepole and jack pine seedlings across all treatments. Error bars represent the 95% confidence interval. Bars with non overlapping error bars are significantly different from each other

volatiles after a 6-week inoculation period. Unfortunately, they did not measure 3-carene, and the only significant difference they found was a strong reduction in α-pinene emission in fungal inoculated lodgepole pine. Direct comparison of the two studies is difficult since they did not quantify their results but only compared ratios. During our experiment, the fungal inoculated lodgepole pine seedlings always released more α-pinene than the controls. Resin monoterpenes from ponderosa pine including α- and β-pinene, 3-carene, limonene, and terpinolene have anti-fungal properties (Himejima et al., 1992). Monoterpenes and other chemical compounds in the resin of host pine inhibit the growth of bark beetle-associated fungi in the southern US coniferous system (Bridges, 1987; Klepzig et al., 1996). There is growing evidence that plants respond to microbial attack by releasing volatiles, which initiates defense mechanisms by signaling within as well as possibly between plants (Kesselmeier and Staudt, 1999). Volatile organic compounds released from inoculated plants induce the resistance to fungi in *Arabidopsis* (Kishimoto et al., 2006), but little is known about plant communication in conifers (Heil and Karban, 2009). The increased emission of 3-carene after fungal inoculation in our study might be due to allocation of that compound towards the inoculation site by the plant, thus fighting the fungus and the wound facilitating its release. In particular, damaged bark can be a major source for VOC emission in conifer seedlings (Heijari et al., 2011).

In the current study, we sampled both volatile and tissue chemistry of both pine species in response to water stress and biological treatments. There are fewer detectable monoterpenes in the seedling volatile profiles compared to monoterpene content in bark and needles. Monoterpene concentration in bark (phloem) and needles also can respond to drought stress (Hodges and Lorio, 1975; Llusià and Peñuelas, 1998). Monoterpenes and resin acids in the woody tissue of Scots pine and Norway spruce seedlings significantly increased as a result of drought exposure (Turtola et al., 2003). In our study, water deficit had no effect on overall monoterpene content in either tissue type. In fact, terpinolene concentration decreased in the bark as a result of water deficit in the lodgepole pine seedlings. The only compound that significantly increased due to water deficit was bornyl acetate in the previous year's bark of lodgepole pine seedlings. The levels of bornyl acetate also was associated with fungal inoculation in jack pine previous year's bark (Fig. 8). Increased bornyl acetate concentrations in the resin of lodgepole pine are associated with Armillaria root disease (*Armillaria mellea*) (Nebeker et al., 1995). Bornyl acetate levels also increase in root and stem tissue of Douglas-fir seedlings (*Pseudotsuga menziesii*) treated with methyl jasmonate, a phytohormone implicated in mediating defense responses (Huber et al., 2005). In some areas, MPB attack is linked to pines infested with *A. mellea* (Tkacz and Schmitz, 1986). Mountain pine beetle antennae do not respond to bornyl acetate (Pureswaran et al., 2004b), but it is possible that it can taste this compound since olfactory and gustatory information are processed in different areas of the insect brain (DeBruyne and Warr, 2006). Gustatory assessment could be way for MPB to evaluate the health status of a host tree and to determine how well defended it is.

The primary goal of our study was to evaluate the chemical profiles of jack pine and lodgepole pine trees and to make reasonable assumptions about jack pine suitability as a potential host to MPB based on its chemistry. All volatile monoterpenes emitted by lodgepole and jack pine seedlings in the study can be detected by the MPB (Huber et al., 2000; Pureswaran et al., 2004b). Some of these host monoterpenes synergize MPB response to the aggregation pheromones *trans*-verbenol and *exo*-brevicomin in trapping studies (Borden et al., 2008). There is strong evidence that myrcene, 3-carene, terpinolene, and α-pinene synergize MPB aggregation pheromone response (Borden et al., 2008). In olfactometer tests, 3-carene was most attractive to MPB out of 20 tested compounds (Conn, 1981). In our study, 3-carene emission increased with plant defense stimulation by *G. clavigera.* This compound might function as an indicator of a weakened tree and could support the aggregation of beetles on susceptible hosts. MPB is able to distinguish between host and non-host volatiles, and may

also use host volatiles to identify weak hosts and avoid the risks associated with trying to overcome tree defenses of a healthy host (Keeling and Bohlmann, 2006). Jack pine emits about three times more 3-carene than lodgepole pine, suggesting that it may be more prone to MPB attacks (Conn, 1981; Borden et al., 2008). Further, jack pine bark contains higher concentrations of α-pinene than lodgepole pine, and α-pinene can be a synergist for the MPB aggregation pheromone, and it is also a precursor for production of *trans*-verbenol, the primary component of MPB aggregation pheromone (Borden et al., 2008).

This study enabled us to compare VOC emission and monoterpene content between lodgepole and jack pine exposed to water stress and different biological treatments under controlled and easy to manipulate conditions. The results suggest that monoterpenes that play a role in pine-MPB interactions differ between lodgepole and jack pine, and also that they are affected by water availability. Shrimpton and Watson (1971) recommended the use of lodgepole pine seedling for studying wound response, since seedlings and mature trees are identical in their resistant response. Nevertheless, inferences made from our study to MPB host use are limited as beetles do not attack trees at the seedling stage. The results from this study, however, are useful for developing the models that guide rational design of field studies, with their inherently greater degree of difficulty and experimental variance. Additional field experiments to understand chemically-mediated interactions among MPB, its associated fungus, and its pine hosts under water deficit conditions are currently being conducted in mature lodgepole, jack and hybrid pine stands. Future studies will assess the behavioral importance of the host monoterpenes from the two species in host detection and acceptance by MPB.

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which in turn affects worker behavior (Richard et al., 2007). Honey bee queens typically mate with multiple males, but the number of matings is highly variable (Tarpy and Nielsen, 2002). Extreme polyandry in honey bees results in greater genetic variability within the colony, which has been associated with healthier colonies (Tarpy and Seeley, 2006), as well as greater productivity and fitness (Mattila and Seeley, 2007). However, according to kin selection theory, lower relatedness among colony members should reduce altruistic and social behavior (Bourke, 1988). Thus, it is expected that queen pheromone composition would reflect her mating history, and that workers should be able to detect these differences. The relationships among multiple matings by the queen, her pheromone composition, and worker behavior have important implications for the evolution of social behavior in honey bees.

The Dufour's gland, previously called the alkaline gland, is an abdominal gland ubiquitous in the Hymenoptera. The gland is associated with the venom, sting sheath, and Koschevnikov glands in the sting apparatus (Martin et al., 2005) (see Fig. 1). Despite several studies, the precise role of the Dufour's gland in honey bees remains unclear. The chemical composition of the gland is affected by caste, task, and age of the bees (Le Conte and Hefetz, 2008). While both queen and worker glands produce hydrocarbons, queens also produce esters (Katzav-Gozansky et al., 1997a). However, the Dufour's gland of workers with developed ovaries also synthesizes queen-like esters (Katzav-Gozansky et al., 1997a), suggesting that the chemical profiles are linked to ovary development and reproductive potential. Previous studies on the effects of mating and reproduction on queen Dufour's gland content have been inconclusive (Katzav-Gozansky et al., 1997b); although newly mated and one-year-old mated queens tended to have lower quantities of Dufour's gland components than virgins, the differences were not statistically significant. Similarly, glands of mated queens tended to contain higher proportions of esters (compared to hydrocarbons) than virgins, but these differences also were not statistically significant. Finally, and contrary to analyses of gland contents, *in vitro* assays of ester and hydrocarbon production by using radiolabeled precursors revealed that mated queens produced significantly higher proportions of hydrocarbons than esters compared to virgin queens. However, these assays likely were confounded by age differences among the queens; other studies have demonstrated that production of queen mandibular pheromone (QMP) varies greatly with age (Plettner et al., 1997).



Fig. 1 Dufour's gland location in honey bees (in red)

The Dufour's gland may serve different functions in different species (Hölldobler and Wilson, 1990). It can produce a trail-marking pheromone, recruitment pheromone, sexual pheromone, or queen control pheromone (Hölldobler and Wilson, 1990; Le Conte and Hefetz, 2008). In honey bees, it has long been debated whether or not the Dufour's gland produces an egg-marking pheromone (Ratnieks, 1995). Selective oophagy of worker-laid eggs (also called 'workers policing') strengthens the functional sterility of their nestmates (Ratnieks and Visscher, 1989). Thus, the switch to queen-like Dufour's gland composition by laying workers may be an attempt to mimic the queen. Indeed, Dufour's gland chemicals have been found on eggs (Katzav-Gozansky et al., 2001). Worker-laid eggs treated with either whole Dufour's gland extracts or the ester fraction—at significantly higher quantities than naturally occurring levels—were removed more slowly than untreated worker-laid eggs, and no difference was observed when eggs were treated with the hydrocarbon fraction (Martin et al., 2002). However, another study that used more natural levels did not demonstrate an effect of Dufour's gland extracts on egg-removal and worker policing (Katzav-Gozansky et al., 2001). Thus, it is unclear whether or not the Dufour's gland in honey bee queens is a significant source of egg-marking cues. However, the Dufour's gland secretions are attractive to workers, suggesting that this gland plays a role in modulating worker behavior and perhaps in colony organization. Attracted workers antennate and groom the queen (Pankiw et al., 1994, 1995), and subsequently spread the queen pheromone through the colony; thus, attraction to the queen plays a role in colony social organization. The Dufour's gland extracts of queens and laying workers are significantly more attractive to workers than extracts of non-reproductive worker bees (Katzav-Gozansky et al., 2001, 2003). Furthermore, the esters, rather than the hydrocarbons, appear to mediate this attraction (Katzav-Gozansky et al., 2003). Finally, exposing bees to both QMP and Dufour's gland extracts has a greater inhibitory effect on worker ovary development than QMP alone (Katzav-Gozansky et al., 2006), suggesting that these chemicals may also serve as a primer pheromone.

Previous studies have demonstrated that chemical composition of the queen mandibular gland is affected by reproductive state and insemination quantity (Richard et al., 2007; Kocher et al., 2008, 2009). Here, we examined the effect of both instrumental insemination and insemination with one or multiple drones on the Dufour's gland. We monitored the attraction of caged workers to the Dufour's gland extracts of virgin, single-drone inseminated (SDI), and multi-drone inseminated (MDI) queens. The chemical profiles of the Dufour's glands of these queens were then characterized by using gas chromatography-mass spectroscopy (GC-MS). We analyzed the quantities and relative proportions of esters and hydrocarbons, since previous studies indicated that mated queens may have lower total quantities of compounds and synthesize higher proportions of hydrocarbons than virgins (Katzav-Gozansky et al., 1997b). Furthermore, since previous studies indicate that the Dufour's gland is linked to ovary development in workers and, thus, might be an indicator of fertility, we also monitored levels of *vitellogenin* expression in the fat body of queens. *Vitellogenin* is an egg-yolk protein precursor, and expression of this gene is higher in recently mated, laying queens than in same-aged virgins (Kocher et al., 2008).

Methods and Materials

General Bee Rearing Colonies headed by SDI queens (*Apis mellifera carnica*, Glenn Apiaries, CA, USA) were maintained at the NCSU Lake Wheeler Honey Bee Research Facility (Raleigh, NC, USA). These colonies were used to produce super-sister queens for the experiments. Due to the haplodiploid sex determination of Hymenoptera, female progeny of SDI-queens have a genetic relatedness of G=0.75. Additionally, colonies headed by naturally mated *Apis mellifera ligustica* or Buckfast-SMR queens (B. Weaver, TX, USA) were used to provide drones for the semen collections and workers for the cage experiments.

Queen Rearing Super-sister queens were produced by grafting young larvae (<24 h) from a single source colony headed by an SDI queen, and rearing them as queens in a queenless colony (Laidlaw, 1977). Capped queen cells were transferred to a dark incubator at 33°C and ~40% relative humidity. One to two days prior to emergence, each capped queen cell was placed into an individual Plexiglas cage (10×10×7 cm) with ~100 1-d-old workers and fed a 50% sucrose solution and honey/pollen paste (45% honey, 45% pollen, 10% sucrose) *ad libitum*. Five days after emergence, some queens (those that were to be inseminated) were treated with CO_2 for 4.0 min (Laidlaw and Page, 1997), while others (those that were to remain virgins) were left untreated. Two days later, queens were treated again with CO_2 for 4.0 min, during which time they were inseminated with semen from either one drone (SDI) or 10 brother drones (MDI) following standard insemination protocols (Laidlaw, 1977). The average drone produces approximately 1 μl of semen, and thus the total insemination volume of each queen was approximately 1 μl for SDI queens and 10 μl for MDI queens. There is obviously variation between different drones, however, and thus volume was not precisely controlled in this study. Note that we are studying the effects of the instrumental insemination procedure (which includes CO_2 treatment, manipulation with the insemination device, and insemination with semen), and the effects of insemination with different numbers of drones. Additional studies on the specific effects of CO_2 and physical manipulation are in preparation. Queens were returned to their respective cages and collected 5 d later onto dry ice and stored at −80°C. A total of 12 virgin, 12 SDI, and 9 MDI queens were produced. Queens were reared in two separate cohorts from the same colony, approximately 1 wk apart (Cohort 1: 7 SDI and 4 MDI; Cohort 2: 12 virgin, 5 SDI, and 5 MDI), but queens from different cohorts were combined in behavioral assays, gene expression studies, and chemical analyses.

Dufour's Gland Extraction Abdomens were dissected in ice-cold RNAlater (Qiagen, Valencia, CA, USA). The Dufour's glands were dissected (Fig. 1) and extracted in 50 μl of pentane containing 0.4 μg/μl of hexadecane (Sigma). Gland extracts were stored at −20°C until they were used for GC analysis or behavioral assays (see below). Internal organs of the abdomen were removed, and the carcass (with attached fat body) was stored at −80°C for gene expression analysis.

Worker Attraction to Dufour's Gland Extracts We compared the responses of adult workers to Dufour's gland extracts of virgin, SDI, and MDI queens. The glands were extracted from each super-sister queen produced as outlined above. Since the internal standard was introduced to each sample, the hexadecane was not expected to contribute to differences in behavioral responses among the sample groups. Previous studies also demonstrated no worker attraction to hexadecane (data not shown). Frames of brood were removed from a single colony headed by a naturally mated queen and incubated at 33°C. One day old workers were collected from the brood frames, and 30 bees were placed into Plexiglas cages (10×10×7 cm). Bees were provisioned with food as described above. Cages were kept in a 33°C incubator with ~40% relative humidity, and manipulations and observations were performed under red light to mimic natural conditions in the hive. In total, 34 cages were maintained for 5 d.

Since worker maturation is altered in the absence of queen pheromone (Morgan et al., 1998; Grozinger et al., 2003), we reared these workers with QMP to mimic natural colony conditions. Bees were exposed to 0.1 queen equivalents (Qeq) of synthetic QMP (Pherotech, Canada). Every day, 10 µl of QMP (0.1 Qeq) in hexane was placed on a glass microscope slide, and the solvent was allowed to evaporate before the slide was placed into the cage. This amount of QMP mimics a live queen in assays of worker behavior and physiology (Slessor et al., 1998, 2005; Hoover et al., 2003).

On the fifth day of the experiment, 30 workers were presented with two microscope slides containing equal quantities of pooled Dufour's gland extract (0.33 Qeq) to compare their responses in binary choices of virgin vs. SDI queens (N= 13 replicates), virgin vs. MDI queens (N= 10 replicates), or SDI vs. MDI queens (N= 11 replicates). The number of workers contacting each slide was counted every 5 min for 15 min after slide presentation. Each cage was only tested once, and the 3 data points per slide were summed as a cumulative measure of worker response.

Data are presented as mean ± SEM. The worker preferences between the two slides of the 34 cages were evaluated with a non-parametric Wilcoxon test.

Chemical Analysis of Dufour's Gland Extracts A 2 µl portion of the 50 µl Dufour's gland extract from each individual queen was analyzed by using GC on an HP 5890 equipped with a capillary DB-5 column (30 m×0.25 mm ID×0.5 µm film thickness, J&W Scientific, Folsom, CA, USA) in splitless mode. Helium was used as carrier gas at a head pressure of 124 kPa (1.3 ml/min). The GC temperature was held at 150°C for 2 min and then increased at 15°C/min to 250°C (5 min), followed by an increase of 3°C/min to 300°C (30 min). Injector and FID were both set at 300°C. We analyzed the Dufour's gland extracts from each of the queens in the study (12 virgin, 12 SDI, and 9 MDI queens).

Compounds were identified using selected samples by splitless capillary GC-MS using a Hewlett-Packard 6890 GC and a model 5973A MSD with an electron impact ion source and an HP-5 ms capillary column (30 m×0.25 mm ID×0.25 µm film thickness). Compounds were identified by using diagnostic ions, comparisons with standard MS databases, and by determination of Kovats indices (Lommelen et al., 2006).

For assessing profile similarity based on the relative proportion of the constituent compounds, a stepwise discriminant analysis was employed using only peaks that were reliably and reproducibly quantifiable (i.e., peaks that were consistently below 0.1% of the total were omitted; Statistica 6.0. StatSoft® Inc.).

Quantification of Vitellogenin RNA Levels by Quantitative Real-time PCR Total RNA was isolated from eviscerated abdominal carcasses (which contained fat bodies) from individual queens using an RNeasy RNA extraction kit (Qiagen, Valencia CA, USA), yielding 0.6–1.5 µg/individual. cDNA was synthesized from 150 ng RNA using Arrayscript reverse transcriptase (Ambion, CA, USA). Abundance of *vitellogenin* transcript was measured using quantitative real-time RT-PCR (qRT-PCR) with an ABI Prism 7900 sequence detector and the SYBR green detection method (Applied Biosystems, Foster City, CA, USA). *eIF3-S8* and *actin* were used as loading controls; both have been used in previous studies of fat body *vitellogenin* expression (Amdam et al., 2006; Grozinger and Robinson, 2007). For each sample, triplicate qRT-PCR reactions were performed and averaged. A standard curve was generated for each primer using dilutions of genomic DNA to calculate the relative quantity of mRNA for each sample. A dissociation curve and negative control (cDNA reaction without RT-enzyme) were used to ensure primer specificity and lack of contamination. *eIF-S8* primers were as in Grozinger and Robinson (2007). *Vg* and *actin* primers were as in Amdam et al. (2006)

We evaluated *vitellogenin* expression in 9 virgin, 10 SDI, and 9 MDI queens. For each individual sample, the ratio of the expression level of *Vg* to that of the geometric mean of the quantities of both reference genes (*eIF3-S8* and *actin*) was calculated. For graphical representation, the samples were normalized to the mean levels in virgin queens. Data are presented as mean ± SEM. The expression level of *vitellogenin* was evaluated with a non-parametric Kruskal-Wallis test, followed by a Mann-Whitney *U*-test.

Results

Worker Attraction to Dufour's Gland Extracts Worker attraction to the Dufour's gland extracts of virgin, SDI, and MDI queens was tested in cages containing 5-d-old bees. Out of 30 bees only a small fraction were attracted by to the different Dufour's gland extracts, which is consistent with previous studies (Slessor et al. 1988; Richard et al., 2007). Worker bees simultaneously exposed to two different Dufour's gland extracts were more attracted to the gland extracts from SDI and MDI queens than to extracts from virgins (t=5.5, P=0.005 and t=1, P=0.027, respectively, Fig. 2), and in a binary choice, they preferred MDI extracts to SDI extracts (t=6, P=0.016).

Insemination Quantity Affects the Relative Proportion of Compounds in the Dufour's Gland The queen Dufour's gland contains a complex mixture of hydrocarbons and esters. Fifty seven compounds were identified, 32 of which were found at relative proportions greater than 0.1% of the total (Table 1). These 32 peaks were used for all of the

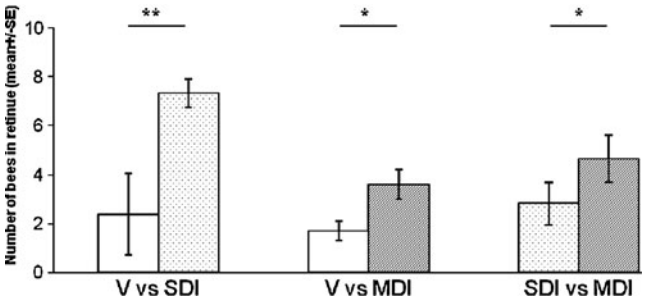


Fig. 2 Worker attraction to Dufour's gland extracts is affected by insemination and insemination quantity. Caged workers were exposed to Dufour's gland extracts of two different queens simultaneously, and the retinue response to each extract was monitored. Worker attraction was compared with a non-parametric Wilcoxon test. (*: $P<0.05$; **: $P<0.01$). *V* Virgin, *SDI* single-drone inseminated queen, *MDI* multi-drone inseminated queen

following analyses. We performed a forward stepwise discriminant analysis to determine if there was significant variation in the relative proportions of these compounds associated with insemination quantity. We also demonstrated a separation between virgin, SDI, and MDI queens ($F_{24,38}$=4.77, $P<10^{-3}$; Fig. 3). All three groups exhibited different chemical profiles (Mahalanobis distances, $P<0.01$).

Insemination Quantity Affects the Quantities of Hydrocarbons and Esters in the Dufour's Gland The total combined quantity of hydrocarbons and esters in the Dufour's glands in all three groups was compared; there were significant differences in total hydrocarbon and ester quantities ($F_{2,32}$= 2.1, $P<0.01$). Pairwise comparisons of the three groups revealed that virgin queens had higher quantities of hydrocarbons and esters than MDI queens (Least Significant Difference, LSD, $P<0.05$; Fig. 4), while SDI queens had intermediate amounts and were not significantly different from either virgin or MDI queens (LSD, $P>0.05$).

The total quantity of hydrocarbons and esters, tested by class, followed a similar pattern. When all three groups (virgin, SDI, and MDI) were compared separately, there were also differences in quantities (hydrocarbons, $F_{2,32}$= 4.23, $P<0.05$; esters, $F_{2,32}$=7.63, $P<0.01$). Pairwise comparisons revealed higher quantities in virgin than in MDI queens (hydrocarbons, LSD, $P<0.05$, esters, LSD, $P<0.05$) while SDI queens had intermediate levels for both hydrocarbons and esters, which were not different from total hydrocarbons in virgin and MDI queens (LSD, $P>0.05$).

Insemination Quantity Differentially Affects the Relative Proportions of Hydrocarbons and Esters in the Dufour's Gland The relative proportion of hydrocarbons in the Dufour's gland among virgin, SDI, and MDI queens revealed differences across the three groups ($F_{2,32}$=3.67, $P<0.05$). Pairwise comparison revealed a higher relative proportion of hydrocarbons in SDI queens than in virgin queens (LSD, $P<0.05$). However, the relative proportion of the hydrocarbons in MDI queens was intermediate and not significantly different from either virgin or SDI queens (LSD, $P>0.05$).

The relative proportion of esters in the Dufour's gland among virgin, SDI, and MDI queens revealed differences across the three groups ($F_{2,32}$=3.67, $P<0.05$). Pairwise comparisons revealed a lower relative proportion of esters in SDI queen Dufour's glands than in virgin queens (LSD, $P<0.05$). However, the relative proportion of esters in Dufour's glands of MDI queens were intermediate and not significantly different from either virgin or SDI queens (LSD, $P>0.05$).

Effect of Insemination Quantity of Vitellogenin Expression We used qRT-PCR to measure *vitellogenin* expression in the queens' eviscerated abdomens. *Vitellogenin* expression was higher in virgin than in MDI queens, using *eIF-S8* and *actin* as loading controls (H(2, N= 29)=10.78, P=0.004; Fig. 5). *Vitellogenin* expression was intermediate in SDI queens and not significantly different from that in either virgin or MDI queens.

Discussion

We instrumentally inseminated honey bee queens with semen from one or 10 drones and compared them to uninseminated virgin queens. These studies allowed us to examine the effects of the instrumental insemination procedure (which includes CO_2 treatment, physical manipulation, and insemination substance) as well as insemination quantity (via the comparison of the SDI and MDI queens) on Dufour's gland composition and gene expression under controlled environmental conditions. Our results clearly demonstrate that both the instrumental insemination procedure and insemination quantity alter queen Dufour's gland composition, which results in differences in worker behavior. Honey bee workers were more attracted to Dufour's gland extracts from inseminated queens than from virgin queens, and to extracts of MDI queens than to SDI queen extracts. Moreover, the overall comparison of Dufour's gland chemical profiles revealed a significant separation between virgin and inseminated queens and between SDI and MDI queens. A small number of esters and hydrocarbons were in higher proportions in MDI queens, while others were significantly lower. It remains to be determined which of the Dufour's gland constituents are most important for eliciting worker attraction to Dufour's gland extract in honey bee queens. Finally, we found a decrease in *vitellogenin* gene expression in MDI queens compared to

Table 1 Chemical composition of honey bees Dufour's gland contents in differentially inseminated queens: virgin, single (SDI), and multi (MDI) drone inseminated queens

	Virgin	SDI	MDI	Kruskal-Wallis H(2,*N*=33)	
Hydrocarbons					
Alkanes					
Pentadecane*	tr	tr	tr	NS	
Heptadecane	tr	tr	tr	NS	
Octadecane*	tr	tr	tr	NS	
Nonadecane*	tr	tr	tr	NS	
Eicosane*	tr	tr	tr	NS	
Heneicosane	+	+	+	NS	
Docosane	tr	tr	tr	NS	
Tricosane	+++	+++	+++	NS	
Tetracosane	tr	tr	tr	NS	
Pentacosane	+++	+++	++		*P*=0.006
Hexacosane	tr	tr	tr	NS	
Heptacosane	++	+++	++	NS	
Octacosane*	tr	tr	tr	NS	
Nonacosane	++	++	++	NS	
Triacontane*	tr	tr	tr	NS	
Untriacontane	++	+++	+++	NS	
Tritriacontane	tr	tr	+	NS	
Alkenes					
Tricosene	+	+	+	NS	
Pentacosene (2 isomers)	++	++	++	NS	
Heptacosene (3 isomers)	+	+	tr	NS	
Nonacosene (3 isomers)	+	+	tr	NS	
Hentriacontene (3 isomers)	++	++	++	NS	
Tritriacontene (2 isomers)	++	++	++	NS	
Alkynes					
Pentacosyne	tr	tr	tr	NS	
Methylalkanes					
9-, 11-, 13-, and 15-Methylpentacosane*	tr	tr	tr	NS	
11-,and 13-Methylheptacosane	tr	tr	tr	NS	
11-, 13-, and 15-Methylnonacosane	tr	tr	tr	NS	
13-, and 15-Methylhentriacontane	tr	+	tr	NS	
DiMethylalkanes					
3,9 Dimethylpentadecane	tr	tr	tr	NS	
Esters					
Tetradecyl dodecanoate	++	+	+		*P*<0.001
Tetradecyl tetradecanoate	++++	+++	+++		*P*=0.007
Tetradecyl (Z)-9-hexadecenoate+Tetradecyl (Z)-11-hexadecenoate	++	++	++	NS	
Tetradecyl hexadecanate+Hexadecyl tetradecanoate	++++	++++	++++		*P*=0.05
Tetradecyl (Z)-9-octadecenoate	++++	++++	++++	NS	
Hexadecyl (Z)-9-hexadecenoate	++	++	++	NS	
Hexadecyl hexadecanoate	+++	++	++	NS	
Hexadecyl (Z)-9-octadecenoate	++	+++	++	NS	
Octadecyl (Z)-9-hexadecenoate	+++	+++	++++		*P*=0.01
Octadecyl hexadecanoate	+	++	+	NS	

The results are presented as relative proportions: tr, traces; +, 0.5–1%; ++, 1–5%; +++, 5–10%; ++++, <10%. * = compounds which were not used in the analysis

virgin queens. However, additional studies are necessary to examine the effects of specific aspects of the instrumental insemination protocol (CO_2 anesthetization, physical manipulation of the reproductive tract).

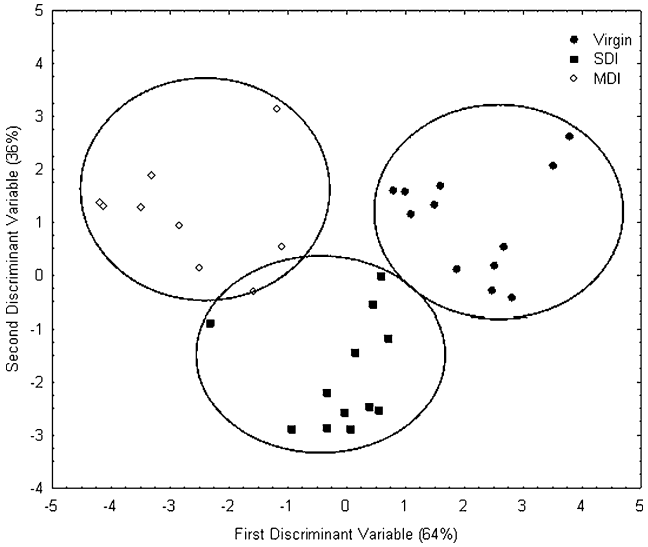


Fig. 3 Insemination quantity significantly alters the chemical profile of the Dufour's gland. Chemical composition of Dufour's gland extracts of virgin, single-drone inseminated (SDI), and multi-drone inseminated (MDI) queens were analyzed using gas chromatography. Discriminant analysis was used to determine if there were significant differences in their Dufour's gland profiles, based on the relative proportion of each compound ($F_{24,38}$=4.77, $P<10^{-4}$). Mahalanobis distances, $P<0.01$. Ellipses were drawn to emphasize the categories, but have no specific statistical meaning

It is important to note that significant changes in the chemical composition of both the Dufour's and mandibular glands occur relatively rapidly, within 5 days after insemination (Richard et al., 2007). While these changes

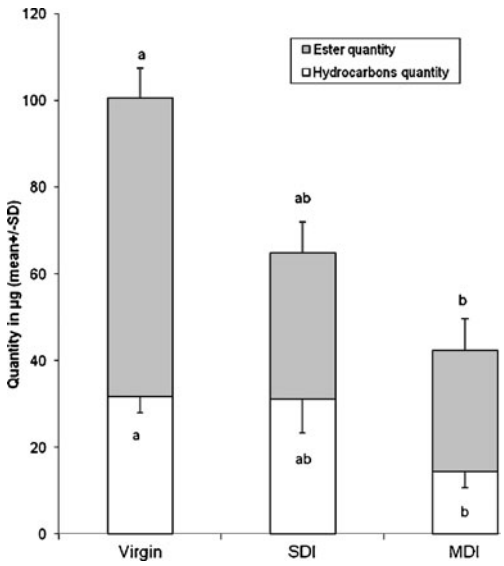


Fig. 4 Insemination quantity significantly alters contents of the Dufour's gland. The quantity of esters and hydrocarbons are significantly different among virgin, single-drone inseminated (SDI), and multi-drone inseminated (MDI) queens (respectively: Kruskal-Wallis: $H_{2,32}$=7.63, p<0.01; $H_{2,32}$=4.23, $P<0.01$)

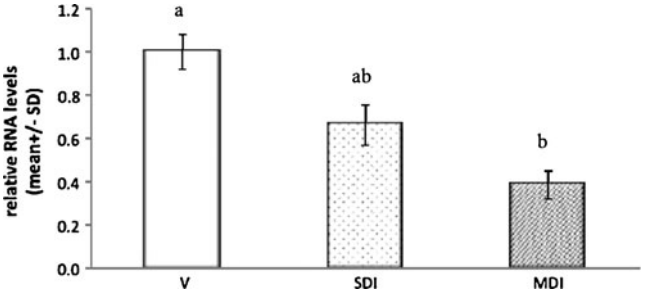


Fig. 5 Expression of *vitellogenin* in the fat body is affected by insemination quantity. Expression of *Vg,* using *eIF3-S8* and *actin* as controls, was monitored using quantitative real-time PCR using individual bees [virgin: $N=9$; single-drone inseminated (SDI): $N=10$; multi-drone inseminated (MDI): $N=9$]. Bars with different letters are significantly different (Kruskal-Wallis: $H_{2,29}$=10.79, P=0.004)

could be due to artificial aspects of the instrumental insemination protocol (such as treatment with CO_2), studies with naturally mated queens have demonstrated that mandibular gland pheromone profiles begin to change before behavioral changes are observed (Kocher et al., 2008). In other insect species, stretch receptors in the bursa, oviducts, or spermatheca, or seminal proteins in male semen are involved in stimulating post-mating changes in females (Sugawara, 1979; Wolfner, 2002, 1997). Both types of mechanisms appear to be operating in honey bee queens (unpublished data), but it remains to be determined if these rapid changes in pheromone production and gene expression are triggered by mechanosensory pathways, chemosensory pathways, or both. Recent studies suggest that insemination volume is an important cue that triggers immediate changes in pheromone production, while semen or seminal fluid may trigger behavioral and brain expression differences over a longer time scale (Kocher et al., 2008; Richard et al. unpublished). However, we note that our MDI queens differed from SDI queens in two regards: they were inseminated with a 10-fold greater volume than SDI females, and they received semen from more genetically diverse sources (ten drones rather than a single drone). Therefore, our experimental design does not allow us to separate the effects of semen volume, sperm number, quantity of semen constituents, or other possible effects of genetic diversity.

While mating stimuli, such as stretch receptors or seminal proteins, may play a role in triggering changes in Dufour's gland composition, the pathways that translate these stimuli into physiological changes in the queen remain to be determined. We observed a quantitative decrease, but not statistically significant, in both *Vg* gene expression and Dufour's gland content in MDI compared to SDI queens, suggesting that these two phenotypes may be regulated by a common pathway. In *Bombus* and *Apis,* the Dufour's gland size and secretion activity in workers

increase with ovary activation (Abdalla et al., 1999a, b). In bumble bees, ovary activation is positively mediated by juvenile hormone (Bloch et al., 2000), but the role of JH in honey bee reproduction is not fully understood (Robinson and Vargo, 1997). In fact, *Vg* expression and JH levels are negatively correlated in honey bees (Pinto et al., 2000; Guidugli et al., 2005; Corona et al., 2007). Thus, while JH may mediate both Dufour's gland secretion and ovary activation in bumble bees (reviewed in Abdalla and Cruz-Landim, 2001), it is likely that an alternative mechanism is operating in honey bees.

The total quantity of compounds in the Dufour's glands decreased following insemination. This decrease may be simply an artefact of the instrumental insemination process. Since our study used same-aged queens, the changes in gland contents are directly linked to the effects of insemination on queen physiology. There are several possible explanations for why insemination would cause a reduction in gland quantities. A high quantity of compounds in the Dufour's gland of virgin queens could be involved in other aspects of reproduction, such as attracting drones during mating flights. Alternatively, the chemical profiles of virgins may deter workers from attacking the queen (see Gilley et al., 2003; Tarpy et al., 2004).

Assuming that instrumental insemination (which includes CO_2 treatment, physical manipulation, and the introduction of semen) mimics natural mating, a third hypothesis is that there is a negative impact of mating on the physiology of the queen. Indeed, in *Drosophila* females, mating stimulates an immune response, suggesting that the introduction of novel male-specific proteins or sperm has effects on the females. Baer et al., 2006 found that newly mated queens of leaf-cutting ants up-regulated their immune responses 9 days after their mating flight. Mating also could be metabolically costly because the female needs to activate processes involved in sperm storage and maintenance of live sperm in the spermatheca. For example, expression levels of antioxidant enzymes increase in the spermathecae of mated queens compared to virgins, suggesting that the mated queens must produce an additional repertoire of proteins to increase sperm longevity (Collins et al., 2004). The cost of mating could explain the short-term effect that we observed before long-term reproductive success. Indeed, we also observed a reduction in *vitellogenin* levels after insemination, despite the fact that *Vg* is necessary for egg-maturation, which suggests that inseminated queens may be negatively affected by mating. Alternatively, there may be a period of physiological remodelling after mating, before egg-production can be fully stimulated. In naturally mated queens (Kocher et al., 2009), these changes occur quickly, while in inseminated queens, changes may occur more slowly.

Previous studies have compared Dufour's gland compounds among non-reproductive virgin, newly mated, and one-year-old mated queens without finding a significant difference in the relative proportion of hydrocarbons and esters (Katzav-Gozansky et al., 1997b). However, analysis of the biosynthesis of Dufour's gland constituents have revealed that the relative proportion of newly synthesized hydrocarbons was significantly higher in mated queens than in virgins, while for esters the pattern was reversed (Katzav-Gozansky et al., 1997b). We found that the relative proportion of hydrocarbons in inseminated queens is significantly higher than in virgins. However, when the three groups of queens were compared separately, hydrocarbon levels of MDI queens were intermediate between the SDI and virgin queens. Thus, insemination appears to have a significant effect on the relative proportions of the hydrocarbons and esters, but insemination volume seems to have additional effects.

Interestingly, workers can discriminate between the Dufour's glands of queens in different mating states. They were more attracted to the gland extracts of inseminated queens than virgin queens, and more attracted to the glands of MDI queens than SDI queens. Previous studies found that queen-like esters, rather than hydrocarbons, seem to play an important role in eliciting this behavior. However, in our studies, the proportion of esters was *lower* in the more attractive blends; thus, the attractive cue may be more specific than a simple increased quantity or proportion of all esters. Some esters were more represented in MDI queens, while others were significantly lower in MDI queens. Similarly, significant differences were seen in individual hydrocarbon compounds.

In summary, both the instrumental insemination procedure and quantity have profound impacts on queen physiology, queen worker interactions, and gene expression in honey bees. Importantly, workers are more attracted to both mandibular gland (Richard et al., 2007) and Dufour's gland extracts (present study) from MDI queens compared to SDI or virgin queens. These behavioral differences are elicited by changes in the chemical profiles that appear to signal the reproductive state of the queens to the workers. Our results suggest that post-mating changes in honey bee queens involve complex processes that are regulated by multiple parameters.

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adaptations, according to species. These include a hardened cuticle (sclerotized or mineralized), protective setae, hard roof-like or wing-like projections (tecta) that protect vulnerable articulations, special body forms such as ptychoidy, in which legs can be retracted to form a sphere of seed-like appearance (Sanders and Norton, 2004; Schmelzle et al., 2009), and even jumping capabilities (Wauthy et al., 1998). In addition, strong claws might give some protection, by preventing detachment from the substrate and thereby avoiding presentation of the vulnerable ventral region (Heethoff and Koerner, 2007). Some of these morphological and behavioral adaptations appear to be effective against small predatory mites (Peschel et al., 2006; but see also Schneider and Maraun, 2009), but have been assumed to have limited value against larger predators such as amphibians (Norton and MacNamara, 1976), ants (Masuko, 1994), and a specialized group of oribatid mite-feeding staphylinoid beetles, the Scydmaenidae (Mollemann and Walter, 2001).

Still, most large arthropod predators in soil seem to consume oribatid mites rarely, or not at all, and this may be related to chemical defense. Most species (the 'glandulate' Oribatida) possess a pair of exocrine oil glands, technically the opisthonotal glands or latero-abdominal glands (Fig. 1), on the hysterosoma ('abdomen'). These have been long assumed to have a defensive function (Oudemans, 1916), although other biological functions, such as lubrication, osmoregulation, or thermoregulation also have been hypothesized (Raspotnig, 2010 and included references). Oribatid oil gland secretions may contain a mixture of aromatics, terpenes, hydrocarbons, and alkaloids of which, collectively, more than 100 specific compounds have been reported (Raspotnig, 2010; Raspotnig et al., 2011a, b). The precise biological function of these compounds remains mostly unstudied. Raspotnig (2006) found that exposure to hexane-soluble compounds from oil gland secretions of the oribatid mite *Collohmannia gigantea* were repellent to the scydmaenid beetle *Euconnus (Tetramelus) oblongus*. In addition, some compounds of oribatids function as alarm-pheromones that initiate escape behavior (Shimano et al., 2002; Raspotnig, 2006). In all other cases, literature reference to secretions being 'pheromones' or 'allomones' is based only on indirect inference.

Considering the abundance of glandulate oribatid mites and their potential predators, knowing the biological function of gland secretions seems crucial for a sound understanding of trophic interactions in soil food webs. The absence of any direct experimental investigation is probably due to the small size of oribatid mites (mostly in the range of 0.2–1 mm), which renders it difficult to test the function of glands by manipulation, such as emptying content or inhibiting discharge.

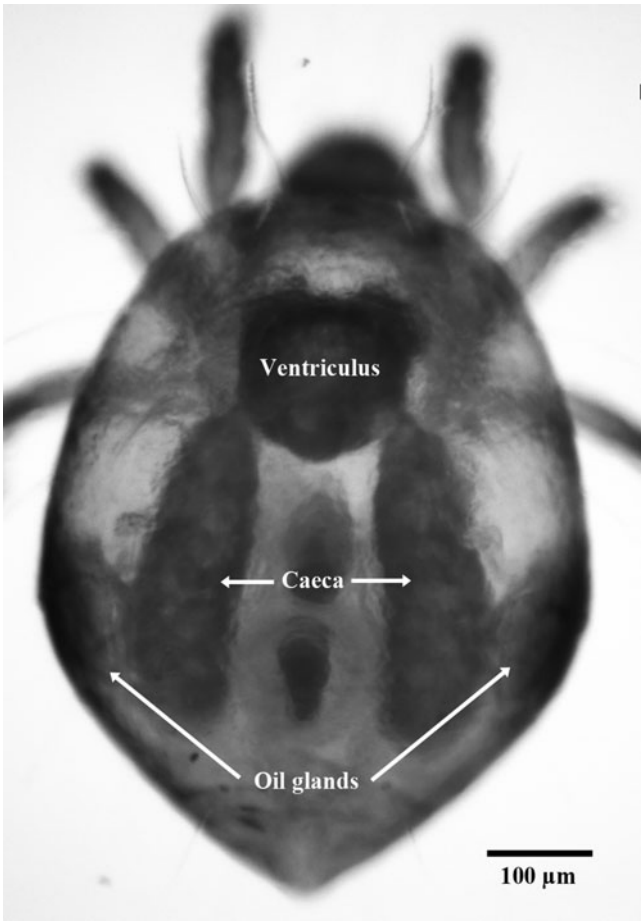


Fig. 1 Micrograph of *Archegozetes longisetosus* showing large oil glands and reservoirs. Image taken under very bright light in phosphate buffered saline to enable view of internal organs

Here, we established a protocol to chemically 'disarm' the oribatid mite *Archegozetes longisetosus* by manipulative emptying of the oil glands, and showed that oil gland secretions, rather than morphological properties, effectively protect the mites from large (~35 times greater body mass) predaceous rove beetles of the genus *Stenus*. This is the first predator–prey bioassay experiment with oribatid mites that tests the function of oil gland secretions, and the first evidence of the adaptive value of these secretions for predator defense.

Methods and Materials

Animals Archegozetes longisetosus was chosen as prey because it is a model chelicerate, and the best-studied oribatid mite (Heethoff et al., 2007; Alberti et al., 2011 and included references). In particular, the oil gland secretions of *A. longisetosus* are well characterized, comprising a set of terpenes, aromatics, and hydrocarbons, the so-called 'Astigmata-compounds' (sensu Sakata and Norton, 2001;

Heethoff and Raspotnig, 2011; Heethoff et al., 2011), which are considered synapomorphic for the glandulate Oribatida (including the clade known as Astigmata; Sakata and Norton, 2001). Moreover, the glands are of special interest for understanding mite evolution and phylogeny (Norton, 2007). Adults of *A. longisetosus* also have limited morphological defenses, including a lightly sclerotized cuticle and moderately long setae that might help to keep some predators at a safe distance. Adult specimens from the laboratory strain of *A. longisetosus* (Heethoff et al., 2007) were cultured as described in Heethoff and Raspotnig (2011).

Two species of the rove beetle genus *Stenus* were chosen as predators, because they are polyphagous and feed mainly on small spiders, mites, aphids, springtails, flies and hymenopterans (Betz, 1998). Besides using the mandibles for prey capture, *Stenus* bears a highly modified labium that can be rapidly protruded, like a harpoon, for catching prey over a distance of up to several millimeters. Using the labium, the prey is attacked without any *a priori* tactile information about quality. Betz et al. (2009) showed that *Stenus* will even attack dummies (plastic heads of insect pins) with the labium, as long as the dummies are moving and are of appropriate size. Specimens of *S. juno* Paykull ($N=23$) and *S. clavicornis* Scopoli ($N=6$) were collected from the reed zone of a small pond near Tübingen (Germany; 48°31′30.74″ N; 9°00′46.53″E), and kept in the laboratory in plastic boxes on a moist mixture of plaster of Paris and charcoal, and allowed to feed on live springtails. Beetles were starved for 5 days prior to the experiments. Experiments were conducted with both species but, due to the low number of specimens, results for *S. clavicornis* were used only for general behavioral comparison with data from *S. juno*, and were not subjected to statistical analysis.

Extraction of Oil Gland Secretion and Gas chromatography–Mass Spectrometry Analyses To determine oil gland contents, control and experimental mites were transferred into 50 µl of hexane for 3 min. to release secretions into the solvent. Gas chromatography–mass spectrometry (GC-MS) analyses and quantification of secretions were performed as described in Heethoff and Raspotnig (2011).

Experimental Discharge of Oil Glands Discharge of oil glands was evoked by dipping live adult mites for 60 sec. into hexane, followed by 1 hr of recovery (with food and water, at 28°C) for the mite, and then a second cycle of 60 sec. in hexane, followed again by 1 hr of recovery. The survival rate from this treatment was >95%, and all specimens discharged their oil glands, with remaining secretions constituting <2% (checked by GC-MS analyses of 100 individuals) of the amount in untreated individuals. There was no difference in moving, feeding, survival, or reproductive rate between hexane-treated and untreated specimens (data not shown).

Bioassays Circular, plastic cuvettes (2.1 cm diam., 2.2 cm height) were used for bioassay-arenas. The floor was covered with plaster of Paris (0.5–1 cm high), which was kept moist during experiments. Fifteen mites, from either the control (fresh mites from the culture) or treated (mites with emptied glands) groups, were placed inside the arena and left for a few min. to acclimate and start moving. An individual *Stenus* was introduced into the arena and observed for 5 min. Mites were replaced (and discarded) and beetles replaced (but retained in individual isolation) after each experiment. Over a period of 3 days, each individual beetle was used for 20 trials, being tested with either control (10 times) or treated (10 times) mites, in random order. Attacks were recorded as successful, if a mite was caught and fed upon, or as unsuccessful, if a mite was released after the attack and survived. Videos were recorded sporadically to demonstrate typical prey capture and feeding behavior (see supplementary video).

Results

Of the 23 *Stenus juno* tested, 10 completely ignored *A. longisetosus* as a food source, regardless of whether mites were controls or treated. The remaining 13 beetles attacked mites from the control group a total of 78 times (28 times with the labium and 50 times with the mandibles; Table 1, Fig. 2). In two cases (both with the same beetle specimen), capture of a control mite was partially successful (outlier in Fig. 2). These mites were caught by the legs or gnathosoma and killed quickly during the attack, which presumably prevented the mites from emitting effective amounts of oil gland secretions. The prey was always immediately released when the beetle manipulated the mites and the mouthparts came into contact with the glandular region on the hysterosoma (see supplementary video). In these two cases of successful capture, the hysterosoma remained almost intact. Remnants of both bodies were extracted in hexane after release from the beetle, and the extracts analyzed by GC-MS: 123% and 184% relative amounts (compared to the internal standard; Heethoff and Raspotnig, 2011) of secretions were observed, compared to the 200–700% relative amounts that are usually observed in secretions of control specimens (data not shown). As the two mites were killed, and a part of their prosoma consumed before the mites were released, these attacks were recorded as successful. Hence, a relative predation success of <3% was recorded for the control group. Generally, contact of the beetle's mandibles or labium with

Table 1 Prey-capturing behavior (number of attacks using the eversible labium or the mandibles for attacking prey) and relative feeding success of *Stenus juno* on *Archegozetes longisetosus* adults with (control) and without (disarmed) oil gland secretions

	Control			Disarmed		
Specimen	Labium	Mandible	Success [%]	Labium	Mandible	Success [%]
1	1	5	0	0	2	100
2	2	5	0	1	4	20
3	2	6	0	0	3	33
4	2	3	0	2	3	20
5	0	6	33	5	10	13
6	1	4	0	1	2	67
7	4	2	0	2	4	17
8	2	3	0	2	3	40
9	3	3	0	3	2	20
10	3	3	0	1	4	40
11	2	6	0	0	11	18
12	2	3	0	1	2	67
13	4	1	0	2	5	29
Sum	28	50	2.6	20	55	28

the glandular region led to intense cleaning of the mouthparts; a direct bite into the oil glands led to a skittish release of the prey, and sometimes a disorientation of the beetle, followed by scrubbing of mouthparts on the substrate (see supplementary video).

The treated mites were attacked 75 times (20 times with the labium, 55 times with the mandibles; Table 1, Fig. 2), with 21 of these mites eaten. This relative feeding success rate of 28% was higher than that of the control group (Wilcoxon rank-sum test, $P<0.01$). After capturing a treated mite, the beetles immediately started to feed, and showed no obvious preference for a starting location. While manipulating the mite, the hysterosoma was not avoided, nor did the cuticular remnants indicate that any body region was preferred or avoided (see supplementary video).

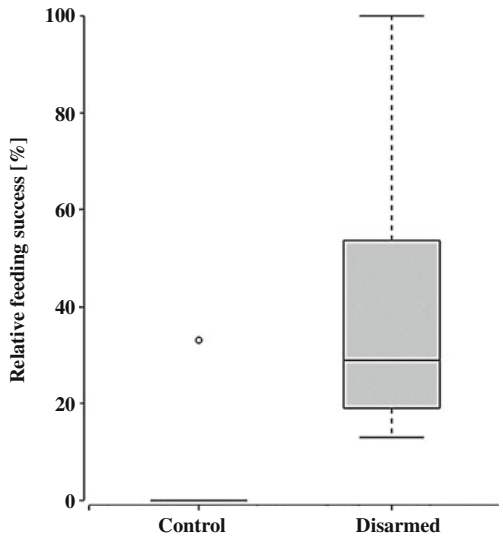


Fig. 2 Boxplot of individual relative feeding success by *Stenus juno* on *Archegozetes longisetosus*. Control = untreated mites taken from culture; Disarmed = hexane-treated mites with empty oil glands. Circle in the control group indicates an outlier

When attacking, the mandibles were used more often than the labium, against both the control ($P<0.05$) and the treated ($P<0.01$) prey groups. There was no difference in how these two capture strategies were applied to prey type (control vs. disarmed: $P>0.2$ for labium; $P>0.5$ for mandibles).

Stenus clavicornis beetles behaved in a similar way with respect to prey capture, handling, and general feeding behavior. Mouthpart-cleaning behavior after contact with the glandular region also was similar. Although the low number of specimens and attacks does not allow meaningful statistical analysis, the trend is similar to that for *S. juno* (2 of the 6 specimens ignored the mites as a prey; 4% and 27% of successful feeding in the control group and the disarmed group, respectively).

Feeding on springtails, a major prey of *Stenus* in nature, was observed and recorded (see supplementary video) for comparison. There were no apparent differences between how springtails and disarmed mites were attacked or eaten (a detailed overview of prey capture and feeding behavior of *Stenus* spp. is given in Betz, 1998).

As capture strategy was essentially the same, behavioral differences of beetles to control and treated mites first became obvious after the attack. Springtails and treated mites were consumed immediately after capture; the beetles remained motionless most of the time during feeding, occasionally turning the prey using the mandibles (see supplementary video). In some cases, the prey was released to the ground after some minutes of mastication and then taken up again with the mandibles for further feeding.

Cuticular remnants of consumed mite specimens were mostly torn to pieces. In contrast, mites from the control group were released immediately if attacked on the hysterosoma. If mites were captured by the legs or the gnathosoma, the beetles started to feed or remained motionless (without feeding) with the prey held in the mandibles. In these cases, mites were released after a few seconds, and beetles cleaned their mouthparts afterward. Mites were released immediately in all cases that they were turned in such a way that the beetle's mouthparts came into contact with the glandular region of the hysterosoma, and this was followed by intense cleaning of the beetle's mouthparts.

Discussion

Chemical defense is a widespread protective strategy among arthropods (Eisner, 2003). While best studied in insects, chemical defense is also well known from chelicerates (e.g., Eisner et al., 1961), with most of this work focused on opilionids (Roach et al., 1980; Raspotnig et al., 2010). Our experiments demonstrate, for the first time, that oil gland secretions of oribatid mites provide protection from predation by arthropod predators.

Predation on Oribatid Mites Peschel et al. (2006) showed that adults of the oribatid species *Eupelops plicatus*, *Steganacarus magnus*, and *Damaeus riparius* were completely rejected as food by the predatory gamasid mite *Pergamasus septentrionalis*. Furthermore, *Achipteria coleoptrata*, *Nothrus silvestris*, and *Liacarus coracinus* also seemed to be well protected (respectively, 71, 57, and 43% of tested individuals survived 30 days of continuous exposure to *P. septentrionalis*). The authors concluded that adult oribatid mites live in an 'enemy-free space', and attributed this to morphological defenses, particularly to the hardened cuticle. Unfortunately, the role of oil gland secretions was not explored in that study, though these secretions are present in many of the tested prey species. *Steganacarus magnus,* like all members of the Phthiracaridae, does not possess oil glands, and its protection from predation probably derives only from a mineralized cuticle and its ptychoid body form. *Damaeus riparius* and *E. plicatus* possess only very small, perhaps vestigial, oil glands that probably contain little secretion (Günther Raspotnig, unpublished data). However, both species have a hard cuticle, and a waxy cuticular covering (cerotegument) or tecta covering vulnerable articulations. These defenses may be sufficient for protection from *P. septentrionalis*, but possibly not from larger predators (as also discussed in Peschel et al., 2006), though direct evidence is lacking. Nothing is known about the oil gland chemistry of *A. coleoptrata*, but secretions of *N. palustris* (and also *N. silvestris*) contain high amounts of geranial, which acts as an alarm pheromone, at least in the former species (Shimano et al., 2002), and possibly as a defensive allomone. Oil gland secretions of *L. coracinus* may contain several acids and their glycerol esters, but these probably function as anti-wetting agents rather than as allomones (Raspotnig and Leis, 2009). However, the high amount of caprylic acid found in *L. subterraneus* might serve as an allomone, as was demonstrated for similar acids for the whipscorpion *Mastigoproctus gigantaeus* (Eisner et al., 1961).

As some of the species investigated by Peschel et al. (2006) produce putative defensive secretions, and as these mites succumbed only after a delay of some days, we suggest that *N. silvestris* and *L. coracinus* were eaten only after they were depleted of chemical defenses. The mites were presumably attacked repeatedly over the course of several days, and the production of secretions may not have been fast enough to counter constant exposure to predators over such a long period. Chemically disarming these species and repeating the experiments with *P. septentrionalis* would test this hypothesis.

Masuko (1994) reported specialized predation on oribatid mites by two species of the ant genus *Myrmecina*. Oil gland composition data are available for only one of these species, *Rhysotritia ardua* (Raspotnig et al., 2011a). This mite produces chrysomelidial, which is known as a defensive secretion of the oribatid *Oribotritia berlesei* (Raspotnig et al., 2011a). Predation on *R. ardua* was inferred by finding remains of this species in the refuse dumps of field nests of *M. flava* and was not observed directly; it was rejected when offered as direct food. Hence, it remains to be elucidated if and how these ants can overcome the chemical and morphological defenses of *R. ardua* (it also has a ptychoid body form; Schmelzle et al., 2009).

Scydmaenid beetles are well-known predators of oribatid mites (Mollemann and Walter, 2001). These beetles typically lift oribatid mites and cut off their legs before turning the mites to start feeding on the gnathosoma (Mollemann and Walter, 2001). Their prey consists of numerous oribatid mite species, some of which possess large oil glands. It is unlikely that scydmaenids are not affected by the oribatid secretions, as Raspotnig (2006) reported escape behavior, intense cleaning of antennae, and narcosis or death following exposure of the scydmaenid *Euconnus (Tetramelus) oblongus* to components from oil gland secretions of *Collohmannia gigantea*. Perhaps, instead, these scymaenids inhibit expulsion of the oil gland secretions, as do predaceous larvae of the beetle *Phengodes laticollis* when feeding on the millipede *Floridobolus penneri* (Eisner et al., 1998)?

In recent years, oribatid mite oil gland secretions have been discussed in a different ecological context. Numerous poison frogs seem to feed specifically on oribatid mites and use their pumiliotoxins and other lipid-soluble alkaloids as 'cleptotoxins' to increase the toxicity of their own skin (Rodriguez et al., 2010; Raspotnig et al., 2011b). In this case, the defensive function of oil gland secretions was functionally sequestered, with the frogs profiting. It is tempting to speculate that the frogs can actually detect oribatid mites through the oil gland secretions (e.g., through taste or olfaction), and hence use these secretions as kairomones.

Stenus *as a Model Predator for Oribatid Mites.* Several species of the genus *Stenus* have been investigated for prey capture and feeding behaviors. Betz (1998) defined three phases in the behavioral sequence associated with preying on springtails: orientation, approach, and prey capture. When attacking *A. longisetosus*, *S. juno* and *S. clavicornis* exhibited all three phases, and no differences were observed during preying on chemically defended or disarmed mites. The rare behaviors of moving sideways while approaching the prey or touching the prey with the antennae, as described for some other *Stenus* species (Betz 1998), were not observed.

Betz (1998) found that use of mandibles by *S. juno* increased with prey size: use of the labium was preferred when attacking small springtails (about 0.5–0.8 mm), while the labium and mandibles were used equally for capturing larger springtails (about 1.2–1.5 mm). As *A. longisetosus* has an intermediate size of 0.9–1 mm, we expected that either the labium would be used preferentially, or both attack types would be used equally. However, we found that *S. juno* used the mandibles more often than the labium. This might be explained by the relative speeds of the respective prey. While springtails are fast moving and can escape by jumping, *A. longisetosus* move slowly and may not be able to escape an attack by the mandibles of *S. juno*. This hypothesis is supported by the fact that *S. juno* uses its mandibles more frequently when feeding on slow-moving aphids (Betz, 1998). The relative feeding success of *S. juno* using the labium was 100% on small, and slightly above 25% on large, springtails. In contrast, when using the mandibles, feeding success was reduced to about 30% and almost 0% in small and large springtails, respectively (Betz, 1998). This is in the same range as the relative feeding success reported here on disarmed (treated) *A. longisetosus*.

Oil Gland Secretions of Archegozetes longisetosus**.** *Archegozetes longisetosus* produces 2-hydroxy-6-methylbenzaldehyde (2,6-HMBD), neral, geranial, neryl formate, γ-acaridial (3-hydroxybenzene-1,2-dicarbaldehyde), tridecane, pentadecene, pentadecane, heptadecadiene, heptadecene, and heptadecane in the oil glands (Heethoff and Raspotnig, 2011 and references therein). The hydrocarbons probably are important as solvents and carrier agents, but some also might have an allomonal function. Most of the compounds have been demonstrated to act as allomones in other arthropod species. Raspotnig (2006) showed that 2,6-HMBD, neral, geranial, neryl formate, tridecane, and pentadecane repel the predaceous scydmaenid beetle *Euconnus (Tetramelus) oblongus.* The aromatic γ-acaridial is specific to oribatid and astigmatid mite oil gland secretions. Its behavioral function is unknown, but it could have an allomonal effect.

Chemical Defense of Oribatid Mites We have shown here for the first time that oil gland secretions of oribatid mites can have an adaptive value for chemical defense against large arthropod predators. We also have shown how other reports of predation on oribatid mites can be viewed in the context of both morphological and/or chemical defense. Taking all evidence into account, we conclude that the 'enemy-free space' hypothesis for adult oribatid mites (Peschel et al., 2006) is supported, but that the importance of chemical defense against predators by oil gland secretions has been underestimated. It is unlikely that cuticular compounds (soluble in hexane and, therefore, likely removed by our extractions) on the mites were responsible for protection, as our GC-MS analyses of extracts showed no compounds other than those from the oil glands. In addition, *Stenus* beetles avoided only the glandular region of untreated mites, and not other cuticular regions.

Of course, 'enemy-free space' is not 'free' to acquire. We do not know the energetic costs of forming morphological defenses or of maintaining a supply of oil gland secretions, but each must be significant. As the selective pressure of predation is unrelenting, such costs probably comprise an evolutionary tradeoff against higher secondary production in a group of mites in which most species grow and reproduce slowly (Norton, 2007).

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including *Pseudoroegneria*, *Stipa*, *Koeleria*, and *Festuca* (Callaway et al., 2005b; Pollock et al., 2009) and has also been reported to impact soil microorganisms *in vitro* (Callaway et al., 2005a). Other evidence has demonstrated that *Centaurea stoebe* growth is facilitated by either promoting beneficial fungal interactions (Callaway et al., 2004b) or by avoidance of harmful microbes normally found in its native range of Europe (Callaway et al., 2004a). Thus, important plant-microbe interactions could be disrupted by the presence of (±)-catechin in soil. However, research to date has not attempted to elucidate the effects that catechin has on specific soil microbial populations or the microbial community in general, nor the specific mechanisms by which it might exert such effects.

To understand how catechin affects soil and root zone microbial communities, we investigated its overall impact on bacterial communities from Romanian (native range) and Montanan (both invaded and non-invaded) soils, as well as on individual soil bacterial isolates (populations) from these same soils. Additional experiments examined the effect of various catechin concentrations on bacterial growth kinetics, as well as the effect of removing catechin from the system in these inhibition studies. The relationship between catechin concentration and its impact on various bacterial soil isolates is discussed.

While there has been a tendency to rely heavily on molecular techniques to examine microbial community phenomena in recent years, with the exception of 16S rRNA gene classification of isolates, we employed simple culture-based most-probable-number (MPN) assays and growth kinetics studies to establish that (±)-catechin has a general suppressive effect on the culturable aerobic, heterotrophic soil bacterial community in all soils. Further, we showed that this suppression is manifested at the population-level as dose-dependent bacteriostatic (i.e., reversible) inhibition that varied between the populations analyzed. These results suggest that catechin exuded by spotted knapweed will have differential effects at the bacterial population-level, which may interfere with or enhance important plant:microbe or microbe:microbe interactions and thus play a role in the invasive success of this aggressive noxious weed.

Methods and Materials

Bacterial Growth Media and Chemicals R2 broth (Difco, Detroit, MI, USA) was prepared according to the manufacturer's suggested protocol and, after autoclaving and cooling to 65°C, amended to a final concentration of 300 μg ml^{-1} cycloheximide (to inhibit fungal growth) by addition of 10 ml of a 30 mg ml^{-1} stock solution (in 100% ethanol) to 1 l R2 broth. R2A plates were prepared in the same fashion as R2 broth, except that 15 g Bacto-agar (Becton Dickinson, Sparks, MD, USA) were added prior to autoclaving. Where minimal medium plus glucose was employed, MMO minimal salts medium was prepared as described previously (Stanier et al., 1966) and amended with 500 μg·ml^{-1} glucose and 300 μg ml^{-1} cycloheximide. Whenever (±)-catechin was amended to liquid or agar media for use in community- and population-level analyses, these were prepared as described below.

(±)-Catechin hydrate (>99% purity) (hereafter referred to simply as catechin) was obtained from Shivambu International (New Delhi, India), and all other chemicals were obtained from Sigma (St. Louis, MO, USA). Due to its limited solubility in water, stock solutions of catechin (150 mg ml^{-1}) in 100% methanol were made fresh for use in community- and population-level inhibition experiments (see below). All liquid and agar media were stored in the dark prior to and during use in experiments. Liquid and agar media amended with various concentrations of catechin were made by adding (after autoclaving and cooling the media to 65°C) the appropriate volume of catechin stock solution, along with the appropriate volume of 100% methanol, so that a constant volume of methanol (20 ml l^{-1}) was added to all media, and only catechin varied in concentration between 0 and 3,000 μg ml^{-1}. This was done to normalize for any methanol-based effects on bacteria, although these are unlikely since the methanol was assumed to have evaporated from media prior to use in experiments.

Source of Soil Samples and Soil Microorganisms Soil was obtained from a native grassland plant community including spotted knapweed in Timisesti, Romania (47.13°N/26.29°E) to represent the soil microbial community in the native range of *C. stoebe* and was collected on March 24, 2005. This sample was comprised of 5 individual soil samples (0–10 cm depth from the A horizon) from random locations within the grassland plot that were composited, mixed thoroughly, and sieved (2 mm) to provide a representative sample hereafter referred to as "native". The soils at this site are classified as Entic Hapludolls and Chernozems. Two different soils (both collected in mid-June of 2005) from western Montana were used to represent invaded and non-invaded soil microbial communities, respectively. Soil from the Bandy Ranch, near Ovando, Montana (47.06°N/113.26°W), classified as Perma gravelly loam, was collected, pooled, and sieved as described above to represent a typical, heavily-infested, spotted knapweed-invaded site, hereafter referred to as "invaded". Similarly, soil from the Clearwater Game Range, near Clearwater Junction, Montana (47.08°N/113.04°W), classified as a Winkler gravelly loam, was collected, pooled and sieved to represent a non-invaded Montana soil that has

never experienced spotted knapweed invasion, nor been treated to prevent it (M. Thompson, *personal communication*). This sample is hereafter referred to as "non-invaded". Multiple bacterial isolates representing phenotypically diverse (based on colony morphology) populations were obtained from each soil type using R2 broth and R2A agar as described below for use in population-level catechin inhibition experiments.

Two other Montana soil samples: one from the root zone of spotted knapweed plants within the Bob Marshall Wilderness (47.80°N/113.03°W) collected on April 6, 2005; and the other from the root zone of spotted knapweed plants on Waterworks Hill near Missoula, Montana (46.88°N/113.98°W) on April 5, 2005 were the source of additional catechin-sensitive and -resistant root zone bacterial strains. As soil had to be air-dried for shipment at 4°C from Romania to the USA, all other soil samples were also air-dried and held at 4°C prior to analysis for consistency between samples. Soil samples were sent to the Soil, Water, & Forage Testing Lab (College Station, TX, USA) for typical soil chemical analysis, the results of which are reported in Table 1.

Pure culture isolates were recovered from the root zone of knapweed plants at the Waterworks Hill and Bob Marshall Wilderness sites via initial growth on R2A+ cycloheximide (300 μg ml^{-1}) and subsequently restreaked onto R2A to ensure purity. Sixteen pure isolates were shown to represent diverse genera and species based on 16S rRNA gene affiliation (see below), and were used to test for catechin sensitivity and recovery from inhibition. Of the sensitive strains, *Burkholderia* strain G-5 was selected for use in detailed growth kinetics experiments to further elucidate the bacteriostatic effects of catechin.

Identification of Bacterial Isolates Based on 16S rRNA Gene Affiliation To ensure the phylogenetic diversity of the isolates used for catechin sensitivity and recovery assays, their 16S rRNA gene was PCR amplified, sequenced, and compared to known 16S rRNA genes by using standard techniques. Briefly, PCR was performed using the generally conserved 16S rRNA gene primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′), 536f (5′-CAGCMGCCGCGGTAATWC-3′), 907r (5′-CCGTCAATTCMTTTRAGTTT-3′), and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) as described previously (Holben et al., 2004). PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and the manufacturer's recommended protocol, and then were sequenced by using the Sanger dideoxy method at the University of Montana Murdock Molecular Biology Facility. The near full-length sequences obtained (i.e., positions 27–1492 based on *E. coli*) were compared with their closest phylogenetic matches based on S_{ab} scores determined using the Sequence Match function of the RDP II website (Cole et al., 2005).

Community-Level Inhibition of Bacteria by Catechin To examine general inhibition of the total cultivable bacterial community by catechin, non-invaded soil was used to enumerate the fraction of the total bacterial community capable of aerobic, heterotrophic growth on general medium in the presence, absence, and following removal of catechin. The numbers of bacteria that grew in R2 broth (containing cycloheximide) with or without catechin were determined by using the three-tube most-probable-number (MPN) approach (Banwart, 1981). To achieve this, non-invaded soil was split into four equal proportions (11.23 g dry weight each) and placed into four sterile 50 ml screw-top flasks. All four subsamples were hydrated drop-wise with sterile double distilled water (ddH_2O) to 40% water holding capacity. These were amended subsequently with stock solutions of catechin in 10 ml of 10% methanol to yield final soil concentrations for catechin of 500, 1000, and 3000 μg g^{-1}. Control treatments with 0 μg g^{-1} catechin were similarly amended with 10 ml of 10% methanol for the reason described above. All treatments were brought to 100% water holding capacity following catechin addition. After 8 days of incubation in the dark at room temperature, 500 mg aliquots from each flask were added to 4.5 ml of

Table 1 List of soils and soil characteristics

Soil	pH	Conductivity (umho/cm)	NO_3^- ppm	P ppm	K ppm	Ca ppm	Mg ppm	S ppm	Na ppm	OM %
Bandy Ranch[a]	6.0	344	135	36	342	2054	213	22	80	5.05
Romania[b]	6.4	444	85	10	485	2962	420	18	70	7.11
Clearwater[c]	5.5	313	112	31	229	1642	187	28	69	4.30
Bob Marshall[a]	6.5	340	119	36	495	2254	266	26	59	4.52
Waterworks Hill[a]	5.0	356	110	57	301	1710	209	16	59	6.18

[a] Invaded Montanan soil

[b] Native Romanian soil

[c] Non-invaded Montanan soil

R2 broth amended with either the same concentration of catechin that was present in the original incubation (0, 500, 1000, or 3000 μg ml^{-1}, respectively) to simulate continuous exposure to catechin, or with no catechin to assess the effect of its removal from the system. These suspensions also contained 1% sodium pyrophosphate and were mixed vigorously by vortexing to aid in detaching cells from soil particles. Aliquots of each of these suspensions (100 μl) then were serially diluted into 900 μl of the same medium (but without pyrophosphate), with vigorous mixing after each dilution. After 7 d of incubation at room temperature with orbital shaking at 150 rev min^{-1} in the dark, each set of tubes was scored for bacterial growth based on visual examination for increased turbidity. Only tubes that exhibited good growth (increased turbidity clearly observable by the naked eye) were considered positive for these tests.

Population-Level Inhibition of Bacteria by Catechin To compare the degree to which catechin inhibited individual aerobic, heterotrophic bacterial populations, the three soils (native, invaded, and non-invaded) were sampled using serial dilution plating to obtain individual isolates from each site. To achieve this, 10 g of soil were placed into 90 ml sodium phosphate buffer (100 mM, pH 7.0), shaken vigorously by hand for 2 min, after which large particles were allowed to settle for 1 min. Decadal serial dilutions into phosphate buffer were performed to 10^{-8}, after which 100 μl of each dilution were spread onto R2A plates supplemented with cycloheximide. All plates were incubated at room temperature in the dark for ~14 d, after which 140 to 150 phenotypically distinct colonies (based on colony size, color, texture, and rate of growth) from appropriate dilutions (producing between 30–300 colonies per plate) for each soil type were sequentially replica-plated onto R2A agar containing: 500 μg ml^{-1} catechin; 1,000 μg ml^{-1} catechin; 3,000 μg ml^{-1} catechin; and, finally 0 μg ml^{-1} catechin to confirm growth of the transferred colony in the absence of catechin (each plate also contained 300 μg ml^{-1} cycloheximide). Colonies were transferred by touching the chosen colony with the blunt end of a sterile, plain wood applicator (Fisher Scientific Pittsburgh, PA, USA Cat# 01–340) and then touching each of the above four plates in succession in an ordered array—termed 'replica plating' in microbiology. All plates were incubated at room temperature in the dark, and colony growth at each position was recorded at 1, 2, 4, and 6 d to assess colony growth rate as a function of catechin concentration. Only colonies that grew successfully on the final control plate (i.e., no catechin added) were scored on the catechin-amended plates. Each bacterial population was categorized as either Not Affected (NA—colonies grew at same rate on catechin-containing plates as on the control plate), Completely Inhibited (CI—colonies only grew on the negative control plate, with no growth on the catechin-containing plates), or Semi-Inhibited (SI—colonies grew on catechin-containing plates, but perceptibly more slowly than on the control plate). Logistic mixed effects models with colonies taken as a random effect were fit by using the restricted maximum likelihood (REML) method to compare populations of microbes from the three sampling locations (Native, Invaded, Non-Invaded) in terms of their sensitivity to catechin at low, medium, and high concentrations (500, 1000, 3000 ppm, respectively).

Assessing Reversibility of Catechin Inhibition To determine whether catechin inhibition was bacteriostatic (and therefore reversible), or bactericidal, nine bacterial strains (representing 7 bacterial genera) that were inhibited by catechin on agar plates were tested for their ability to grow in liquid medium before and after removal of catechin from the medium. All strains were initially grown in MMO medium with 500 μg ml^{-1} glucose to mid-log phase. These cultures then were transferred (1:100 inoculum) into MMO medium containing 500 μg ml^{-1} glucose and the following amendments: 0 μg ml^{-1} catechin; 2000 μg ml^{-1} catechin; and 2000 μg ml^{-1} catechin that was removed after the corresponding no catechin control culture had reached stationary phase. The catechin concentration of 2000 μg ml^{-1} was selected as being intermediate in the previous inhibition experiments. Catechin was removed by collecting the inhibited cells by centrifugation, carefully pouring off the supernatant, resuspending and washing the cells once with sterile PB (100 mM phosphate buffer; pH 7), collecting by centrifugation, then resuspending the cells in MMO medium with 500 μg ml^{-1} glucose without catechin. All treatments were performed in triplicate and incubated at room temperature in the dark with rotary shaking at 150 rev min^{-1}.

Effect of Catechin on Bacterial Growth Kinetics *Burkholderia* strain G-5, a representative catechin-sensitive soil bacterial population, was used to examine in detail the kinetics of growth in the absence, presence, and following removal of catechin from the medium. Growth kinetic data for strain G-5 were obtained by 1:100 dilution of triplicate fresh overnight cultures in MMO medium with 500 μg ml^{-1} glucose into fresh MMO + glucose medium amended with: 0 μg ml^{-1} catechin; 2000 μg ml^{-1} catechin; and 2000 μg ml^{-1} catechin that was subsequently removed. For this last treatment, catechin was removed by collecting the inhibited cells by centrifugation after 36 h (when the parallel cultures of strain G-5 without catechin had reached stationary phase), carefully pouring off the supernatant, resuspending and washing the cells once with sterile PB, collecting by centrifugation, then resuspending the cells in

fresh MMO medium containing 500 μg ml^{-1} glucose but no catechin. All treatments were performed in triplicate and incubated at room temperature in the dark with rotary shaking at 150 rev min^{-1}. Cell growth was monitored based on optical density at 550 nm (i.e., OD_{550}) using a Spectronic 20D+ spectrophotometer (Milton Roy USA, Ivyland, PA, USA). As catechin exhibits limited auto-oxidation with resultant absorbance at 550 nm, two sets of blanks were used for this experiment. The blank for cultures grown without catechin was MMO medium with 500 μg ml^{-1} glucose, while the blank for cultures grown in the presence of catechin was MMO medium with 500 μg ml^{-1} glucose and 2000 μg ml^{-1} catechin. Both blanks were incubated under conditions identical to the cultures. To avoid adding methanol to these cultures, catechin was dissolved completely by gentle heating in 5 ml of sterile ddH_2O and immediate addition to the MMO+glucose medium, effectively diluting the catechin before it could precipitate.

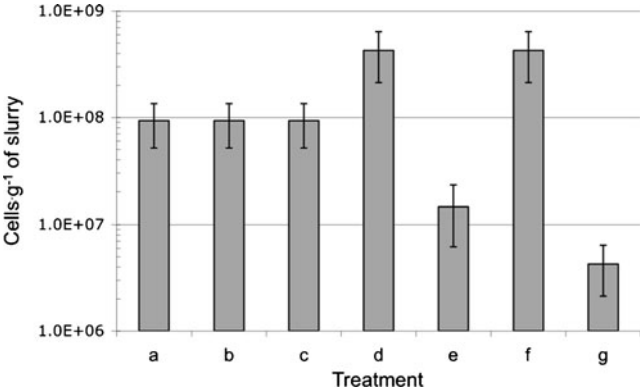


Fig. 1 Most-probable-number (MPN) analysis of non-invaded soil bacterial community grown in R2 broth + cycloheximide medium with and without catechin. Bars represent the average of triplicate determinations ±1 SE. Treatments were as follows: **a**) control (0 μg g^{-1} and 0 μg ml^{-1} catechin in soil and media, respectively); **b**) 500 μg g^{-1} catechin in soil, 0 μg ml^{-1} catechin in media; **c**) 500 μg g^{-1} and 500 μg ml^{-1} catechin in soil and media, respectively; **d**) 1000 μg g^{-1} catechin in soil, 0 μg ml^{-1} catechin in media; **e**) 1000 μg g^{-1} and 1000 μg ml^{-1} catechin in soil and media, respectively; **f**) 3000 μg g^{-1} catechin in soil, 0 μg ml^{-1} catechin in media; **g**) 3000 μg g^{-1} and 3000 μg ml^{-1} catechin in soil and media, respectively

Results

Inhibition of the Soil Bacterial Community by Catechin MPN enumeration using R2 broth was used to assess the general effect of catechin on the aerobic heterotrophic bacterial community of soils. This was accomplished by determining the fraction of the total bacterial community capable of growth in the presence of various concentrations of catechin. The number of culturable heterotrophs ranged from 4.27±2.14 (standard error, SE)×10^8 cells (g of soil)$^{-1}$ with no catechin to as low as 4.27±2.14 (SE)×10^6 cells (g of soil)$^{-1}$ when high levels of catechin were present (Fig. 1). Dose-dependent inhibition of growth with increasing catechin concentration was observed when catechin concentrations were maintained. Interestingly, samples treated with catechin but then released from its presence showed a complete reversal of catechin inhibition, and their MPN values were comparable to or exceeded those observed in the no-catechin control tubes.

Inhibition of Growth of Individual Bacterial Populations by Catechin Having shown that catechin had a general suppressive effect on the growth of the aerobic, heterotrophic bacterial community, the effect of catechin at the level of individual bacterial populations was investigated through ordered replication of colonies on media with various concentrations of catechin or no catechin via replica plating. Higher concentrations of catechin showed greater inhibition in terms of percent of populations capable of growth (Fig. 2). Further, when the growth of each individual bacterial population was classified as being not affected (NA), semi-inhibited (SI), or completely inhibited (CI) relative to no-catechin controls, there were significant differences between native soil populations and the invaded and non-invaded Montana populations based on the restricted maximum likelihood (REML) method ($P<10^{-7}$, $P<10^{-6}$, respectively). There were no significant differences in the normal growth percentages between the invaded and non-invaded Montana populations ($P=0.602$). Separate comparisons were also made within each catechin dose level (Table 2), which upheld the overall difference between the native and Montana populations at all catechin levels except for non-invaded populations at 1000 ppm.

Catechin Inhibition is Bacteriostatic To further investigate the seeming reversal of inhibition that was observed in the MPN analysis, a suite of soil bacterial isolates were tested for sensitivity or resistance to the inhibitory effects of catechin, and the sensitive isolates tested for their ability to resume growth after removal of catechin from the media (Table 3). Of the sixteen isolates tested, seven were unaffected by catechin and, interestingly, six of these were identified as *Pseudomonas* sp. representing five different taxa based on their 16S rRNA gene sequence, while the seventh was identified as *Rhodococcus* sp. (Table 3). Eight of the remaining nine strains tested were completely inhibited by catechin, while one, *Bacillus* strain C-5, was semi-inhibited. Growth of all nine of the inhibited strains (including C-5) was completely restored after removing catechin from the medium by washing the cells and placing

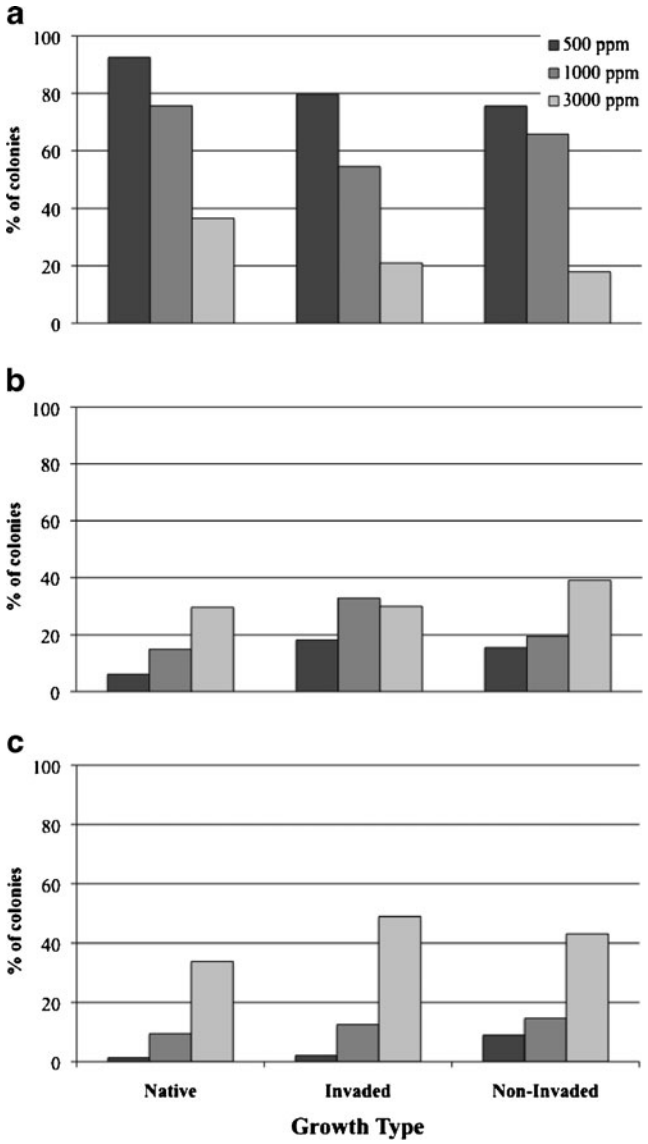


Fig. 2 Bacterial populations from native (148 isolates), invaded (143 isolates), and non-invaded (123 isolates) soils plated on R2A agar containing indicated concentrations of catechin. **a**: Percent of Not Affected colonies (NA); **b**: Percent of Semi-Inhibited colonies (SI); **c**: Percent of Completely Inhibited colonies (CI). Indicated growth of populations was as compared to its growth on the control plate with no catechin

them into fresh MMO plus glucose medium without catechin (Table 3). Seven different genera were represented in the group of nine sensitive—but recoverable—isolates, suggesting that catechin may be a fairly broad inhibitor of growth across bacterial populations, but clearly, not all are affected.

Catechin Effects on Bacterial Growth Kinetics To more closely examine the inhibitory effects of catechin on bacterial growth, *Burkholderia* strain G-5 was subjected to detailed growth kinetics assays in the absence, presence, and following removal of catechin in the medium (Fig. 3). When catechin was present, growth of strain G-5 was essentially completely inhibited relative to the no catechin control, even after prolonged (~200 h) incubation. When catechin was removed from the medium after 36 h of completely inhibited growth (by washing the cells and placing them into fresh MMO plus glucose medium), normal growth kinetics essentially indistinguishable from the no-catechin controls resumed following a substantial (≥80 h) lag period (Fig. 3).

Table 2 Between-soil comparison of catechin effect on soil bacterial populations

Catechin Concentration	Native vs. Invaded	Native vs. Non-Invaded	Invaded vs. Non-Invaded
500 ppm	0.0022[a]	0.0002	0.4216
1000 ppm	0.0002	0.0765	0.0615
3000 ppm	0.0025	0.0009	0.6212

[a] *P* values obtained by pair-wise restricted maximum likelihood (REML) analysis between soils at each catechin concentration.

Discussion

While the impact of catechin on indigenous plant species of North America has been extensively studied (Buta and Lusby, 1986; Iqbal et al., 2003; Inderjit et al., 2008), its effects on soil bacterial communities have not been thoroughly examined. The few reports to date on this topic have suggested that catechin is "antimicrobial" to certain laboratory strains relevant to soils (e.g., *Xanthomonas campestris*, *Agrobacterium radiobacter*, *Erwinia carotovora*, and *Erwinia amylovora*) (Veluri et al., 2004), but have not examined the nature of the inhibitory effect(s) or their generality against a broad array of soil microbial populations.

In the current study, we demonstrated a general dose-dependent suppressive effect of catechin on the aerobic, heterotrophic bacterial community of soils that reduced MPN enumeration values by up to two log units (a 99% decrease) at the highest concentration tested (Fig. 1). The inhibitory effect on total community numbers was fully relieved upon transfer to catechin-free medium, suggesting that its action is bacteriostatic rather than bactericidal. We note that the higher MPN numbers observed following relief from catechin inhibition in this MPN assay (Fig. 1) suggest the existence of some poorly understood rebound effect upon release from catechin suppression, since control treatments that never experienced catechin exposure and the 500 μg ml^{-1} treatment that was not significantly suppressed did not exhibit this same increase.

It is also interesting to note that a small proportion of the bacterial community in these soils (~1% based on the MPN assay) was capable of forming a soluble red compound in the presence of catechin (data not shown). This compound

Table 3 Description of microorganisms and their reaction to catechin

Strain	S_{ab} score (# of bases)	Closest Match (genus level)	Gram reaction	Glucose	Glucose + Catechin	Post- Wash
C-1[a]	**0.913 (1246)**	***Aeromicrobium***	**positive**	**Growth**	**No growth**	**Growth**
C-2	**0.957 (1344)**	***Chryseobacterium***	**negative**	**Growth**	**No growth**	**Growth**
C-4	**0.975 (1325)**	***Arthrobacter***	**positive**	**Growth**	**No growth**	**Growth**
C-5	**0.996 (1397)**	***Bacillus***	**positive**	**Growth**	**Poor growth**	**Growth**
C-6	0.985 (1285)	*Pseudomonas*	negative	Growth	Growth	NT[b]
C-7	0.978 (1443)	*Pseudomonas*	negative	Growth	Growth	NT
C-8	0.984 (1423)	*Pseudomonas*	negative	Growth	Growth	NT
C-9	**0.987 (1222)**	***Flavobacterium***	**negative**	**Growth**	**No growth**	**Growth**
C-10	**0.977 (1230)**	***Arthrobacter***	**positive**	**Growth**	**No growth**	**Growth**
G-1	**0.773 (1412)**	***Pedobacter***	**negative**	**Growth**	**No growth**	**Growth**
G-2	1.000 (720)	*Rhodococcus*	positive	Growth	Growth	NT
G-4	0.995 (1386)	*Pseudomonas*	negative	Growth	Growth	NT
G-5	**0.974 (1382)**	***Burkholderia***	**negative**	**Growth**	**No growth**	**Growth**
G-7	0.995 (1398)	*Pseudomonas*	negative	Growth	Growth	NT
G-8	0.995 (1400)	*Pseudomonas*	negative	Growth	Growth	NT
G-9	**0.968 (1379)**	***Flavobacterium***	**negative**	**Growth**	**No growth**	**Growth**

[a] Strains in bold are inhibited by catechin but their growth is restored when catechin is removed from the medium

[b] NT stands for Not Tested

may represent some catechol-like intermediate of catechin breakdown by bacterial populations in the soil. While this interpretation is consistent with the observations, it is purely speculative in the absence of additional data and the basis for and significance of this phenomenon remain unknown at this time.

The data from the replica-plating assay with individual bacterial populations (refer to Fig. 2) are also indicative of dose dependent catechin inhibition in that, for each soil type, the number of NA populations decreased, while the number of SI or CI populations increased with catechin concentration. Figure 2 also demonstrates that the soil bacterial community from the native range is significantly more resistant to the inhibitory effects of catechin than either the invaded or non-invaded sites in Montana as evidenced by higher numbers of NA populations and lower numbers of SI or CI populations at each catechin concentration (Fig. 2 and Table 2). This phenomenon may reflect long-term selection for catechin-tolerant bacterial populations in the native range of *C. stoebe.*

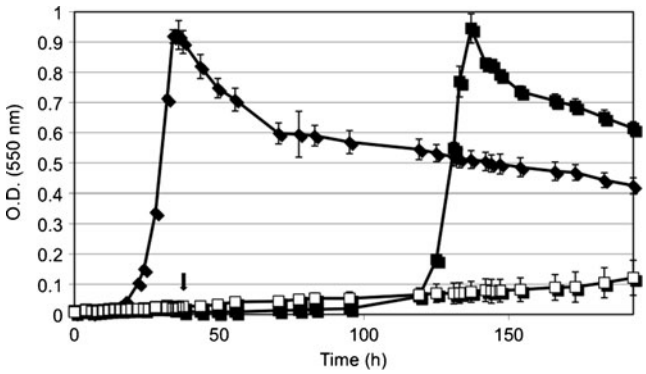


Fig. 3 Growth of *Burkholderia* strain G-5 in the presence, absence or following removal of catechin. Analyses were performed in triplicate and error bars indicate ±1 SE. ♦, MMO + glucose; □, MMO + glucose + catechin; and ■, MMO + glucose + catechin where catechin was removed at t=36 hr (indicated by arrow) and the cells placed into fresh MMO medium with glucose but no catechin. All glucose concentrations were 500 μg ml^{-1} and catechin concentrations were 2000 μg ml^{-1}

These results raise intriguing possibilities regarding soil bacterial population dynamics in the face of invasion by spotted knapweed. We previously have suggested that the soil microbial community from the home range of *Centaurea stoebe* LAM. has stronger inhibitory effects on its growth than soil microbes from where the weed has invaded in North America (Callaway et al., 2004a). The data presented herein suggest that some specific bacterial populations showing no sign of inhibition by catechin at any concentration (NA) have been selected for over time in the native soil such that the community has overall greater resistance or tolerance to the bacteriostatic effects of catechin. By contrast, NA populations in the non-invaded and invaded soils likely have some form of intrinsic resistance or tolerance for catechin and may represent potential successor populations in the presence of introduced catechin, possibly producing a shift in bacterial community composition to populations resistant or tolerant to catechin with prolonged exposure. This scenario could enhance the invasive success of spotted knapweed if those populations either facilitate its growth or suppress growth

of native plant populations. CI populations, on the other hand, represent catechin-sensitive taxa that might be supplanted by resistant or tolerant ones during knapweed invasion, although moderate numbers of CI populations also were detected in the native soil. Such population shifts could have dramatic effects on soil nutrient cycling or other important processes depending on the identities and functions of the inhibited populations (Thorpe and Callaway, 2011).

The SI populations also merit consideration. These were observed from all soils and there may be several reasons for their partial state of inhibition. Among these is the observed instability of catechin over time (Blair et al., 2005, 2006; Perry et al., 2007; Broeckling and Vivanco, 2008; Inderjit et al., 2008). Since catechin effects are dose-dependent, biotic or abiotic transformation of catechin into a biologically inert form might decrease its effective concentration below the inhibitory level for these populations over time, thereby restoring growth. Another possible explanation might be the ability of some microbial populations to mitigate the inhibitory effects of catechin. Since catechin is a known metal chelator (Gomah and Davies, 1974; McDonald et al., 1996; Lim et al., 2005; Pollock et al., 2009), delayed growth may represent a lag period prior to activation of pathways for sequestration of metals or other micronutrients required for growth. Indeed, earlier experiments that examined the effects of catechin and various related analogs (present as natural products in teas, wines, and certain other food products) on gastrointestinal microflora showed that various bacterial populations were differentially impacted by these compounds (Ishigami and Hara, 1993; Hara, 1997). Among those not affected were the Lactic Acid Bacteria, which, interestingly, are reported to have no metabolic requirement for iron (Archibald, 1983; Boyaval, 1989; Bruyneel et al., 1989; Pandey et al., 1994; Imbert and Blondeau, 1998). Finally, since catechin and similar compounds variously have been reported to damage cell membranes (Hoshino et al., 1999), block cellular transport systems (Shimizu et al., 2000; Song et al., 2002), and damage DNA (Hayakawa et al., 1999), bacterial populations with less extensive damage or more robust repair systems may be immune to (i.e., NA), or capable of overcoming with time (i.e., SI), inhibitory effects of catechin. Given the genetic, metabolic, and physiological diversity of soil bacteria, it is perhaps not surprising that a full spectrum of responses to catechin exposure was observed across these populations.

Sixteen soil bacterial populations isolated from the root zone of spotted knapweed and representing nine different genera were tested for their ability to grow in the presence of catechin (Table 3). Nine of these sixteen microbes exhibited either complete or partial inhibition of growth by catechin, which was shown to be restored after its removal. To better illustrate that catechin was capable of fully inhibiting growth and that growth could be fully restored by removing catechin, the kinetics of growth of *Burkholderia* strain G-5, were examined in detail. The results clearly demonstrated complete inhibition of growth of G-5 by catechin, and that normal growth was fully restored following catechin removal. We hypothesize that the lag phase observed after catechin removal (Fig. 3) represents catechin-induced cell damage that required repair before normal growth resumed. The possibility that differential sensitivity within the population could explain the observed lag, with some cells actually killed while others rapidly resume growth upon catechin removal, seems less likely given that no bacterial isolate tested was killed by catechin and every inhibited isolate was able to fully recover from its bacteriostatic effect.

The results presented herein collectively demonstrate that catechin is a bacteriostatic, not bactericidal, inhibitor of some, but not all, bacterial populations in soils. This is ecologically important in this invasive plant system because, if catechin were somehow removed (e.g., through knapweed suppression followed by biodegradation or abiotic transformation), inhibited bacterial populations could potentially rebound and resume normal growth. This scenario may be of significant relevance in attempts to mitigate knapweed invasion and restore the ecology of invaded systems.

In these experiments, we used a range of catechin concentrations ranging from 0–3,000 ppm (i.e., $\mu g\ ml^{-1}$). It is salient to note that reports in the literature have indicated catechin concentrations in soil harboring knapweed that range from 1.1 $\mu g\ g^{-1}$ soil to ~650 $\mu g\ g^{-1}$ soil (Blair et al., 2006; Perry et al., 2007). However, those prior studies measured catechin in bulk soil samples by using a procedure involving extraction and detection of chemically pure catechin by HPLC. We suggest that the concentrations of catechin a bacterium would encounter at the point of exudation into the soil (i.e., in the rhizoplane/rhizosphere/root zone) would be much higher and that the range of concentrations used in the current study are ecologically relevant for microbes living at the root—soil interface. We also suggest that a steep gradient of catechin concentration exists as a function of distance from knapweed roots, as is commonly found for other root exudates (Kuzyakov et al., 2003; Sauer et al., 2006), and that the inhibitory effects of catechin on soil microbial populations thus decrease with distance from the knapweed rhizoplane. We also note that catechin has been reported to be chemically unstable in media, sand, and soil (Blair et al., 2005, 2006; Perry et al., 2007; Inderjit et al., 2008; Pollock et al., 2009), presumably being transformed through biotic and/or abiotic processes into derivative compounds (e.g., the aforementioned soluble red compound), and that such compounds would not be

detected as catechin by the currently available HPLC-based methods. Finally, as we have shown recently, derivative compounds of pure catechin may have greater or less toxicity than chemically pure catechin (Pollock et al., 2009). In other words, it is difficult to determine precisely how much biologically active catechin is present in various compartments of soil ecosystems.

Catechin exuded by *C. stoebe* potentially disrupts a number of important plant:microbe interactions, both beneficial and antagonistic. Such disruptions may play an important role in the invasive success of spotted knapweed, as they impact both the plant and microbial components of the system (Callaway and Ridenour, 2004) by acting at the level of individual soil bacterial populations (this work). Thus, catechin may facilitate the invasion of spotted knapweed into non-invaded soils through a variety of potential mechanisms including suppression of pathogens of *C. stoebe*, facilitation of pathogens specific for native plants, suppression of microbes promoting growth of competitors, or facilitation of microbes promoting growth of *C. stoebe*.

Our results show that a diverse set of bacterial populations from soils native to spotted knapweed, invaded by spotted knapweed, and not invaded by spotted knapweed were differentially affected by catechin. More specifically, the data show that: 1) the inhibitory effect is dose-dependent at both the community and the population level; 2) individual microbial populations are differentially sensitive to catechin; 3) catechin is bacteriostatic to those populations that are affected; and, 4) removal of catechin from the system allows normal bacterial growth kinetics to resume. We hypothesize that, while native systems (including bacterial communities) have adapted to spotted knapweed and catechin over long time periods, the short-term response of an invaded community might be such that removal of catechin by degradation or transformation could allow the invaded bacterial community to recover its normal structure and function. This last suggestion may have profound ramifications for attempts to restore knapweed-infested ecosystems and warrants further investigation.

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Preliminary studies suggested that pea aphids, *Acyrthosiphon pisum* (Harris), elicit minimal plant volatile defense responses in broad bean plants. Herein, we asked whether failure of bean plants to respond to aphid feeding was due to an inability of plants to recognize and or respond to aphids, or alternatively, due to aphids' ability to suppress plant volatile emission. In several plant systems, when insects feed on plant tissue, the plant releases a blend of chemicals that is qualitatively and quantitatively different from control plants (see Felton and Tumlinson, 2008). However, in some cases (Turlings et al., 1998), when aphids feed on broad bean plants they do not induce a change in the emitted volatile blend or at best induce VOCs at a much lesser extent than caterpillar feeding. There are two possible explanations for why aphids do not cause changes in volatile emission. One is that aphids feed primarily intercellularly and thus cause relatively little damage to plant tissue compared to caterpillars and damage fewer plant cells, causing less induction of damage related volatiles. We feel that this is unlikely as aphids do cause considerable tissue degradation at high densities and cause punctures to epidermal, parenchyma, and vascular cells during stylet penetration and feeding (Tjallingii, 1995). Likewise, proteinaceous elicitors have been found within aphid saliva that trigger plant responses (de Vos and Jander, 2009). Therefore, aphids should provide ample cues for a rapid detection and a systemic plant response. The alternative hypothesis is that aphids are able to counteract plant defenses as they feed.

We designed experiments to measure the ability of pea aphids to suppress volatile emissions induced by beet armyworm caterpillars, *Spodoptera exigua*, which cause an increase in volatile emission when feeding on broad bean plants.

Methods and Materials

Plant and Insect Rearing Broad bean plants, *Vicia faba* L. (Var. Windsor) were used for all rearing of aphids and for volatile entrainments. *Vicia faba* seeds were purchased from Territorial Seed Co. (Cottage Grove, OR, USA) and planted singly in 10 cm round plastic pots and watered from the bottom as needed. Plants were grown in a greenhouse under controlled climatic conditions, (16:8 hr, L:D, 25°Celsius, 50% relative humidity) in autoclaved soil (MetroMix 200, Sun Gro Horticulture Canada Ltd, Bellevue, WA, USA).

Pea aphids used in volatile experiments were taken from an in-house colony originating from Lexington, KY, USA (Dr. Grayson Brown, University of Kentucky Department of Entomology). Beet armyworm (BAW) caterpillars, *Spodoptera exigua* were obtained as eggs from Benzon Research Inc. (Carlisle, PA, USA) and were reared on artificial diet until they reached the 3rd larval stage.

Aphid colonies were reared on one to 3-wk-old broad bean plants with the aerial portion of the plants enclosed within air-permeable (185×390 mm) cellophane bags (Armin Zeller, Nachf. Schütz & Co, Langenthal, Switzerland). Colonies were groomed weekly to maintain colony densities that were not crowded, i.e., they did not promote dispersal morph production.

Aphid Feeding The effect of aphid feeding on plant volatile production was tested on each of 8 plants over a 6-d period. Aphids feeding on plants was the sole treatment, and uninfested plants were used as controls. Fifty adult aphids were placed on each treatment plant 1 hr prior to the start of air entrainment. We chose this level of infestation because it did not result in the production of dispersal morphs and did not cause complete death of the plant after 6 d of air entrainment. For this experiment, volatiles were collected from the headspace of plants twice daily; one diurnal collection from 9:30 to 17:30, and one nocturnal collection from 21:30 to 5:30 the next morning.

Aphid and Beet Armyworm Feeding We tested for the effects of simultaneous feeding of aphid and caterpillar on plant volatile production. Treatments consisted of plants with: (1) aphids; (2) caterpillars; (3) aphids and caterpillars; or (4) control plants with no herbivory. We placed 50 adult aphids on plants used for volatile entrainment 24 hr before placing 3 third-instar caterpillars. This was done to establish sustained aphid feeding prior to caterpillar feeding. Aphids and caterpillars were placed on plants at 13:30 hr on their respective days. Caterpillar activity was monitored throughout the daytime collection periods, and new caterpillars were added to maintain even damage over all replicates. Six replicates were performed.

Air Entrainment Air was sampled from the headspace of 2-wk-old *V. faba* plants within individual volatile collection chambers (Fig. 1). The chambers consisted of 11 cm diam by 30 cm tall glass cylinders that were capped with Teflon® lids and placed over aluminum guillotines. Plants were inserted into the chambers so that the pot and soil were below the guillotine and the aerial portion of the plant was contained within the glass cylinder. Cotton wool was wrapped around the base of the stem to prevent aphids or caterpillars from escaping (Fig. 1).

Purified air originating from an in-house compressor was pumped into the chambers at a rate higher than the rate at which it was sampled (1.5 l per min in, 1.0 l per min sampled) to prevent contamination from outside the chamber. As air was removed from the chamber it passed over an adsorbent trap containing 30 mg of Super Q (80/100

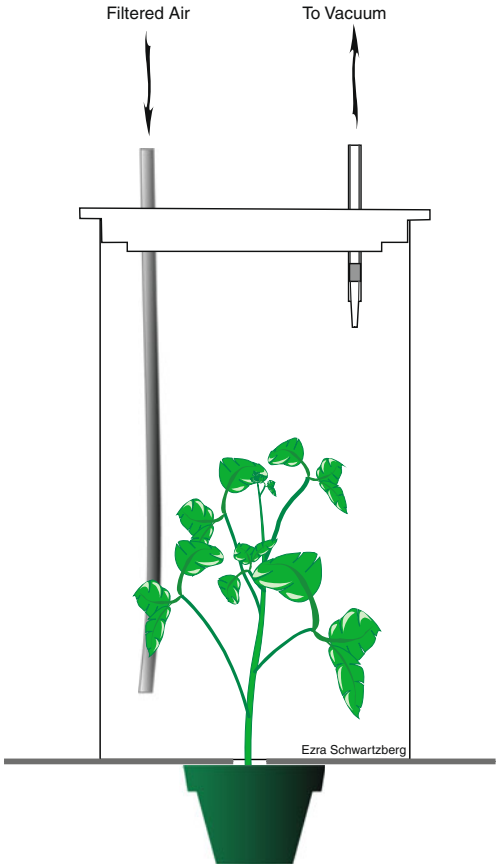


Fig. 1 Schematic of a volatile entrainment chamber. The aerial portion of the plant was isolated within glass chambers fitted with filtered air inlets and vacuum outlets. Headspace volatiles were collected on adsorbent filter cartridges over 8 hr collection periods. The chambers consisted of 11 cm diam by 30 cm tall glass cylinders that were capped with Teflon® lids and placed over aluminum guillotines

mesh, Alltech Inc, Nicholasville, KY, USA). Headspace volatiles were entrained for 8 hr per day during the photophase from 09:30 to 17:30. Volatile entrainments started at 9:30 hr, 4 hr prior to aphid placement on test plants allowing for an 8 hr collection period prior to the placement of caterpillars. Aphids were placed on plants within collection chambers at 8:30 on the morning of the first 8 hr collection period. Caterpillars were placed on plants at 17:30 following the first 8 hr collection period. Diurnal volatile entrainment of plants with aphid and caterpillar feeding continued until the end of the last collection period. At this point, leaves were photocopied to calculate total leaf area consumed between treatments.

Chemical Analysis Absorbent traps were removed within 24 hr of each collection period and eluted with 100 µl dichloromethane. Twenty µl of 8 ng/µl of nonyl-acetate in hexane were added as an internal standard to the elutant from each trap.

All samples were analyzed quantitatively by using a Hewlett-Packard 6890 GC equipped with a flame ionization detector (FID) and fitted with an Equity-1 bonded phase capillary column (0.25 mm×0.25 m×30 m, J&W Scientific, Folsom, CA, USA). The column temperature was programmed with an initial temperature of 40°C for 3 min, followed by an increase to 220°C at a rate of 5°C/min, with a 5 min hold at 220°C. The injector was set at 220°C. Samples were injected splitless, and the split valve was opened 0.75 min after injection. Helium was used as carrier gas at a linear flow rate of 36.3 cm/sec. Because 1/3 of all of the volatile compounds emitted from within the test chamber were lost to the outside environment, the resulting values for volatile emission were multiplied by 3/2 to obtain the total emission of compounds from the plant over each 8 hr collection period.

Selected samples were analyzed using an Agilent 6890N GC coupled with an Agilent 5973N Mass Selective Detector (GC-MS) in the electron impact mode (+70 eV) with an HP-1 bonded phase capillary column (30 m× 0.25 mm×0.25 um, J&W Scientific, Folsom, CA, USA) programmed as indicated above for compound identification. Kovats Indices (Kovats, 1988) were calculated for all peaks and compound mass spectra were compared to the NIST/EPA/NIH Mass Spectral Library (NIST02) for initial compound identification. Additionally, retention time and mass spectra were compared to those of synthetic standards.

Statistical Analysis For comparison of volatile emission, an analysis of variance was performed for each identified compound or the sum of all analyzed compounds per collection period using the statistical software package JMP 5.0 for Macintosh. When the sum of all emitted compounds was calculated, (*E*)-*ß*-farnesene was removed due to its perceived production source, i.e., because (*E*)-*ß*-farnesene is produced by aphids it only adds variation to the sum of plant-emitted compounds (see "Discussion"). All data were $\log_e(x+1)$-transformed to satisfy the assumptions of ANOVA and *post-hoc* comparisons were conducted by using Tukey's honestly significant difference (HSD) test.

Results

Aphid Feeding Aphid feeding had relatively minor effects on host plant volatile production. Over the six-day collection period, aphid colonies grew to the point where their host plant started to become desiccated. At the end of collection, aphid infested plants were noticeably smaller than controls, although there were no significant changes in volatile production. The exception to this was an increased emission of (*Z*)-3-hexenol by aphid-infested plants during the photophase of days three through six, and the increase of (*E*)-*ß*-farnesene after the fourth day of aphid infestation (Fig. 2).

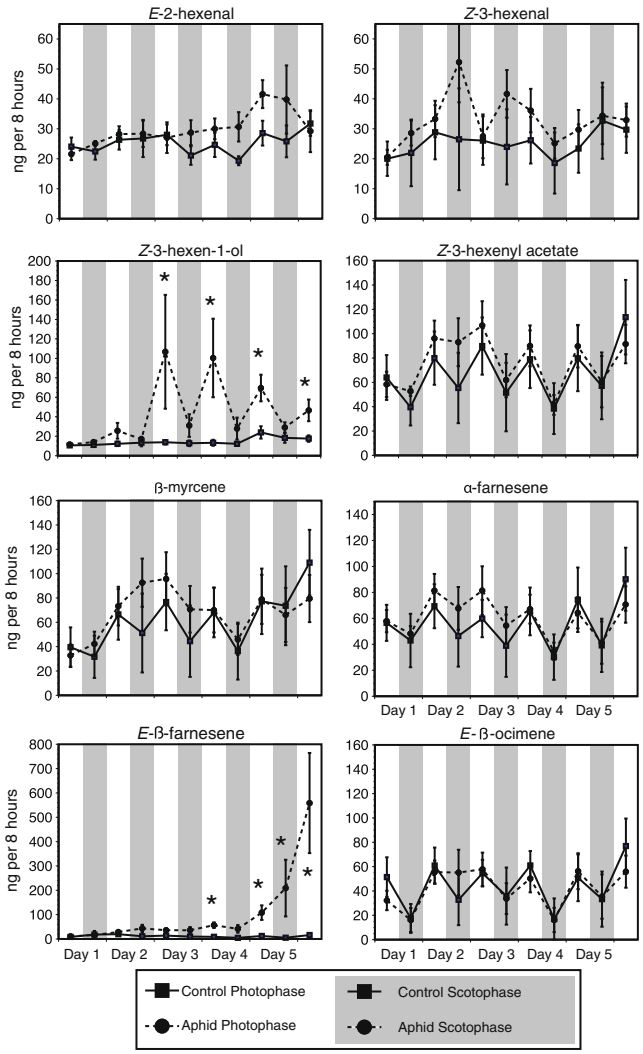


Fig. 2 Major compounds released by broad beans infested with 50 adult pea aphids during the photophase (*light area*) and scotophase (*shaded area*). Data show mean and SE of (*E*)-2-hexenal, (*Z*)-3-hexanal, (*Z*)-3-hexen-1-ol, *β*-myrcene, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, (*E*)-β-farnesene, and *α*-farnesene. Data show values from eight replicates. An asterix indicates significant differences between treatments ($P<0.05$)

Combined Aphid and Beet Armyworm Feeding Beet armyworm caterpillars caused an induction of several volatile compounds within the first 8 hr collection period after the onset of feeding, with the greatest induction of most VOCs after 48 hr of feeding. Among the most abundant BAW-induced VOCs are the green leaf volatiles (GLVs) (Z)-3-hexenol and (*Z*)-3-hexenyl acetate, and the monoterpene (*E*)-*β*-ocimene, the sesquiterpene (*E*)-*β*-caryophyllene, and the homosesquiterpene (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (Fig. 3) (Table 1). In this experiment, aphid feeding did not cause a significant increase in any plant-derived volatile compounds (Fig. 4), including the green leaf volatile (*Z*)-3-hexenol, which was not induced in aphid-infested plants. The lack of emission of this compound is contradictory to the previous experiment (Fig. 2), however, due to inherent variability between tests, this was not seen as abnormal.

Caterpillar-induced volatiles were altered significantly by the presence of aphids (Figs. 4 and 5), and moreover, there were differences in how each compound was affected. For example, *β*-caryophyllene and TMTT were suppressed by aphid feeding. In other words, lesser amounts of these compounds were released from plants when fed upon by the combination of aphids and caterpillars as compared to volatile release from plants in response to caterpillar feeding alone (Fig. 4). The GLV (*Z*)-3-hexenyl acetate and the sesquiterpene (*E*)-*β*-ocimene were reduced by aphid-feeding as compared to controls for at least one of the days sampled (Fig. 4). Moreover, aphid feeding had an affect on total volatile production in broad bean plants. This was seen by a significant reduction in emitted volatiles from plants with aphids as compared to controls on days one, two, and four, ($F=3.99$; $df=3, 23$; $P=0.022$) ($F=10.31$; $df=3, 23$; $P<0.001$) ($F=12.71$; $df=3, 22$; $P<0.001$), respectively, as well as compared to BAW feeding on day three ($F=4.54$; $df=3, 23$; $P=0.01$) (Fig. 5). Finally, we observed differences in (*E*)-*β*-farnesene emission among treatments, specifically, from aphids or the combination of aphids and caterpillars as compared to controls or caterpillars only (Fig. 4).

Leaf area measurements were made at the end of the two volatile collection experiments to assess total leaf area, leaf area consumed, and percent leaf area consumed. Aphid infested leaves were smaller than control plant leaves $t(10)$, $P=0.02$. There were no significant differences in total leaf area, leaf area consumed, or percent leaf area consumed between plants fed upon by beet armyworm caterpillars, or the combination of aphid-infestation and beet armyworm feeding.

Discussion

We observed different responses of broad bean plants to herbivory by two herbivores with differing feeding habits. While broad bean plants responded to herbivory by beet armyworm caterpillars (BAW), seen by an increase in the emission of several green leaf and terpenoid volatile compounds, broad bean did not respond similarly to herbivory by aphids. The volatile profile emission of broad bean plants infested with aphids remained similar to, or even lower than that of control plants. With the exception of the green leaf volatile (*Z*)-3-hexenol, there was no significant increase in emission of volatile compounds, suggesting that, unlike other plants, (Quiroz et al., 1997; Han and Chen, 2002a; James, 2003; Zhu and Park, 2005;

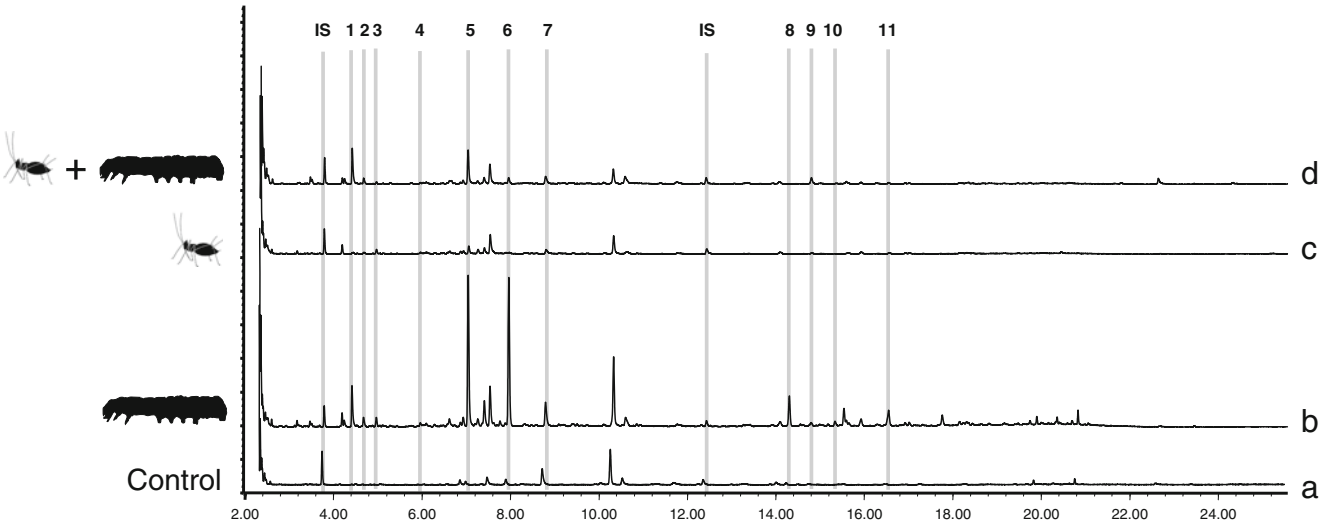


Fig. 3 Chromatograms of volatile compounds emitted on Day 3 from (*a*) control plants, (*b*) plants fed upon by 3 beet armyworm caterpillars (BAW), (*c*) plants colonized by 50 aphids, or (*d*) combination of aphids and BAW. Major compounds are labeled as in Table 1

Harmel et al., 2007), broad bean plants do not respond to herbivory by pea aphids. Our results also were inconsistent with previous studies that examined VOC emission from broad bean plants fed upon by pea aphids (Du et al., 1998). While Du et al. (1998) found that broad bean plants produced several compounds following aphid infestation, including 6-methyl-5-hepten-2-one, (Z)-3-hexenyl acetate, (*E*)-*ß*-ocimene, (*Z*)-3-hexenol, (*E*)-*ß*-farnesene, and geranic acid, we were unable to replicate this in our study. We speculate that these differences in plant behavior are the result of either differential responses inherent to plant variety, research conditions, or aphid strain. It has been demonstrated previously that plant VOC response is affected by both the variety of plant being tested (Loughrin et al., 1995; Gouinguene et al., 2001; Degen et al., 2004), herbivore species within the same feeding guild (De Moraes et al., 1998), and even herbivore life stage (Rodriguez-Saona et al., 2002) (Halloran, personal communication), although difference in induction by different strains of the same insect species has not been documented. We believe the difference is less likely due to aphid strain, as we have tested several strains of pea aphids, including both red and green morphotypes with no noticeable differences in plant VOC emission (unpublished data). A more likely reason for differences between our study and Du and others (1998) is aphid density. Our infestations were

Table 1 Major compounds released from broad bean plants with aphids, beet armyworm (BAW), BAW and aphids, or controls on day 3, 2 d after the start BAW feeding and 3 d after aphid colonization. Data show untransformed means ± SE of values of six replicates. Different letters represent significant difference of the mean at ($P>0.05$)

Compound	Peak	KI[a]	RT[b]	RT[c]	Control	BAW	Aphids	Both
E-2-hexenal	1	831	4.24	5.65	Nq[a]	5.56±3.05[a]	Nq[a]	6.63±4.26[a]
Z-3-hexen-1-ol	2	842	4.42	5.85	8.93±6.05[bc]	37.2±8.36[a]	1.19±5.12[c]	28.98±6.87[ab]
E-2-hexen-1-ol	3	858	4.68	6.12	9.84±4.23[a]	10.89±2.53[a]	3.46±1.57[a]	10.39±2.98[a]
Benzaldehyde/α-pinene	4	932	5.97	7.58	8.77±4.48[a]	6.39±1.81[a]	4.07±2.35[a]	6.46±2.52[a]
Z-3-hexenyl acetate	5	990	7.06	8.73	63.49±20.05[a]	91.18±46.11[a]	9.07±4.98[b]	41.95±17.19[ab]
E-ß-ocimene	6	1041	7.97	9.71	31.23±22.83[ab]	75.4±45.91[a]	0.83±0.54[b]	5.32±2.21[ab]
DMNT	7	1074	8.59	10.37	4.85±2.57[a]	3.17±1.80[a]	0.6±0.60[a]	1.95±0.91[a]
ß-caryophyllene	8	1413	14.30	16.38	2.18±1.35[a]	39.27±12.87[b]	0.3±0.30[a]	0.71±0.51[a]
E-ß-farensene	9	1448	14.81	16.91	0.68±2.73[a]	4.59±3.22[ab]	14.94±5.01[bc]	28.66±7.03[c]
n-Pentadecane	10	1499	15.60	17.72	4.94±1.77[a]	6.92±3.03[a]	0.75±0.54[a]	6.4±3.93[a]
TMTT	11	1567	16.55	18.61	4.26±1.35[ab]	20.01±3.65[a]	0.46±2.51[b]	5.64±3.28[ab]

Superscripts denote: [a] Kovats indices on HP-1 column, [b] Retention time (Equity-1 column) gc-fid, and [c] Retention time (HP-1 column) gc-ms

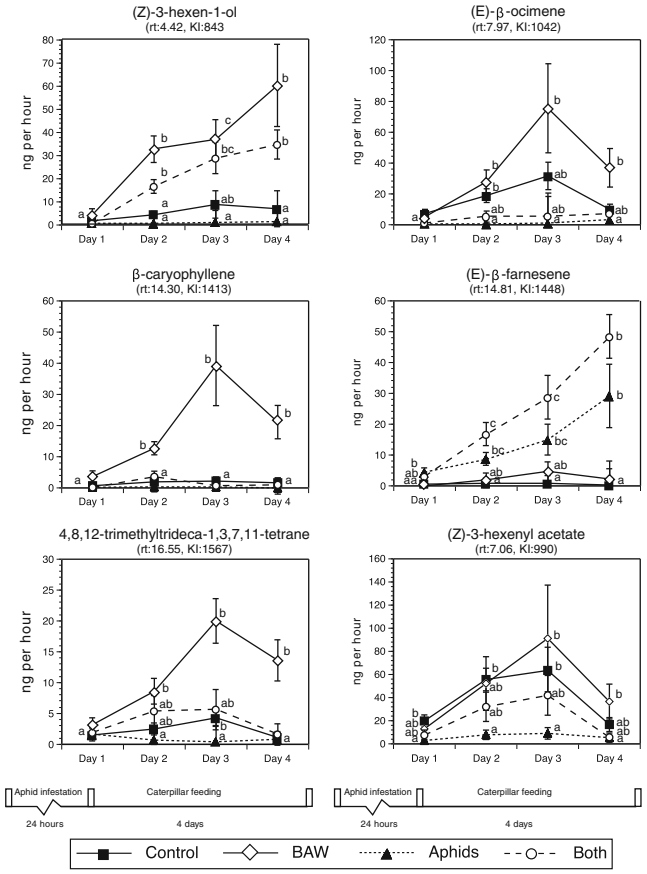


Fig. 4 Major compounds released by broad beans infested with BAW; *solid line*, *open diamond*, aphids; *dotted line*, *solid triangle*, combination of aphids and BAW; *dashed line*, *open circle*, or control; *solid line*, *solid square*. Data show mean and SE of (*Z*)-3-hexen-1-ol, (*E*)-*β*-ocimene, (*E*)-*β*-caryophyllene, (*E*)-*β*-farnesene, TMTT, and (*Z*)-3-hexenyl acetate. Data show values from six replicates. Different letters indicate significance differences between treatments ($P<0.05$)

considerably lower than those of Du and others (1998), and this could explain the observed differences.

The presence of increased emission levels of (*Z*)-3-hexenol may not necessarily be a consistent indicator of aphid infestation. While we report an increase in this compound, we have not seen it consistently produced from broad bean fed upon by aphids (unpublished data).

Broad bean plants responded to caterpillar herbivory by emitting VOCs as compared to control plants or plants infested with aphids. Specifically, BAW caterpillars induced compounds previously shown to increase upon herbivore damage, including (*E*)-*β*-caryophyllene, and (*E, E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (Colazza et al., 2004) as well others not previously reported to be induced by beet armyworm feeding on broad bean. We had expected that broad bean would respond to BAW feeding, and this is not the first study to show differential induction of VOCs from plants fed upon by both chewing and piercing-sucking insects (Rodriguez-Saona et al., 2003, 2005; Delphia et al., 2007). In fact, it is common to see a much greater induction of VOCs from chewing insects as compared to piercing-sucking insects (Turlings et al., 1998).

The ability of aphids to alter the response of broad bean plants to BAW feeding helps to explain a potential mechanism for the reduced VOC responses that are seen among plants fed upon by aphids and other piercing sucking insects as compared to plants fed upon by chewing insects such as caterpillars. While aphids feed on plant phloem, and reach the phloem intracellularly, they do cause considerable damage to plant tissue. As aphids guide their stylets to phloem tissue, they puncture almost every mesophyll cell along the stylet path (Prado and Tjallingii, 2007), and often they exhibit repetitive puncturing of the phloem sieve elements (Tjallingii and Gabrys, 1999). Therefore, we argue that it is unlikely that aphid stylet penetration simply goes un-noticed by plants. In our study, the ability of aphid feeding to suppress VOC emission in response to caterpillar feeding suggests that aphids actively suppress plant responses. Caterpillars fed equally when tested as the sole herbivore, or in combination with aphids, suggesting that the degree of damaged tissue is similar between treatments. Suppression of caterpillar-induced VOC emission eliminates the possibility that plants respond less to aphid feeding simply due to aphids' apparently stealthy feeding habits.

Beet armyworm induced compounds are quantitatively and qualitatively different from those emitted from control plants. For example, the sesquiterpene *β*-caryophyllene is produced only from broad bean plants fed upon by caterpillars and not from control plants, thus demonstrating a qualitative difference in emission. Additionally, the monoterpene

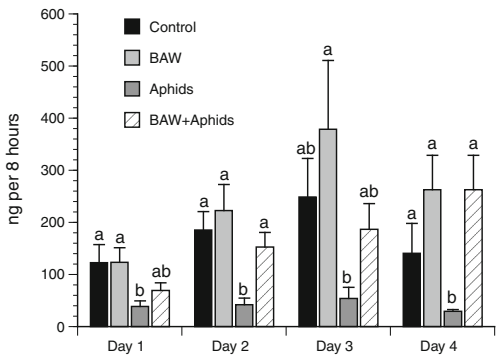


Fig. 5 Total volatiles released by broad beans infested with 3 beet armyworm caterpillars (BAW); *lightly shaded*, Aphids; *dark shaded*, combination of aphids and BAW; hatched, or control; *black bars*. Data show mean and SE of the sum of the 13 most abundant volatiles (with the exception of (E)-β-farnesene). The aphid alarm pheromone, ((E)-*β*-farnesene) was omitted from the calculation of total volatiles due to its suspected origin from aphids rather than the host plant. Data show values from six replicates. Different letters indicate significance differences between treatments ($P<0.05$)

(*E*)-*ß*-ocimene, which is consistently emitted from control plants, increases in emission in response to caterpillar feeding, demonstrating a quantitative difference in emission.

Aphid feeding also has varying affects on emission of different compounds. This is apparent when comparing total volatile emission (Fig. 5) to individual compounds (Fig. 4). In particular, it seems that BAW induced sesquiterpenes are affected more than beet armyworm induced GLVs. For example, the GLV (*E*)-2-hexenal is induced upon BAW feeding and is not affected by the presence of aphids, whereas emission of the terpenoid compounds *ß*-caryophyllene and (*E*)-*ß*-ocimene is greatly affected by the presence of aphids. Terpenes and GLVs are products of different metabolic pathways within plants; terpenes are produced via the mevalonate pathway while GLVs are produced via the oxylipin pathway (Creelman and Mullet, 1997). It is likely that the mechanism(s) responsible for the observed suppression of caterpillar-induced volatile production act differently, depending on what metabolic pathway they are affecting. This observed differential suppression also eliminates the possibility that suppression effects are due to changes in plant size or virus load that result from aphid infestation. Both of these also have been shown to decrease volatile production in a non-discriminatory manner, i.e., by decreasing all plant emitted compounds evenly (Jimenez-Martinez et al., 2004).

The differences in the propensity of various herbivore-induced compounds to be suppressed by aphid feeding also may shed light on the relative importance of individual compounds on parasitoid attraction. The pea aphid parasitoid, *Aphidius ervi*, is attracted to a blend of plant volatiles, including several of the ones emitted by our plants under caterpillar herbivory (Du et al., 1998). Interestingly, aphids effectively suppress some of these compounds, for example, the emission of the parasitoid-attracting compound (*E*)-*ß*-ocimene, thus suggesting that there may be a selective advantage for limiting a plant's production of this attractant. Suppression of spider mite-induced (*E*)-*ß*-ocimene by whiteflies inhibits the attraction of the predatory spider mite *Tetranychus urticae* (Zhang et al., 2009). Pea aphids also suppress the emission of (*E*)-*ß*-caryophyllene, a compound that, depending on its ratio to (*Z*)-3-hexenal, germacrene D, and TMTT, also attracts the parasitoid *Aphidius ervi* (Pareja et al., 2009).

Aphids may benefit from affecting VOC emission in other ways as well. Many aphids rely on the production and perception of the aphid alarm pheromone (*E*)-*ß*-farnesene. However, the response of pea aphids to this alarm pheromone can be affected by the ratio of (*E*)-*ß*-farnesene to sesquiterpenes and other compounds being produced from its host plant. For instance isothiocyanates act as synergists to alarm pheromone for the turnip aphid, *Lipaphis (Hyadaphis) erysimi* (Dawson et al., 1987), while (*E*)-*ß*-caryophyllene acts as an antagonist for the cabbage aphid, *Mysus persicae* (Dawson et al., 1984). The ability of pea aphids to affect the emission of these and other compounds may affect how they respond to natural enemies, and/or crowding, both of which are influenced by (*E*)-*ß*-farnesene perception (Kunert and Weisser, 2003).

In summary, our results show that feeding by multiple herbivores simultaneously affects the induction of VOCs compared to these same herbivores feeding independently. Furthermore, we demonstrate that aphid infestation inhibits the release of several VOCs normally induced upon feeding by the herbivore *S. exigua*, and that the level of suppression incurred by pea aphid infestation varies depending on the compound. While we speculate on why some compounds are suppressed and others are not, we have no definitive explanation why, or how, some compounds are affected differently from others. However, aphids may be actively suppressing inducible plant VOCs that alter aphid behavioral responses to their environment, or the response of aphid natural enemies to the aphid's host plant.

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of the plant response, different populations of a single plant species may provide highly variable targets for prospective herbivores.

For insects that sequester defensive compounds produced by their host plants, variation in these compounds may have substantial consequences for these insects and their interactions with their natural enemies. For example, individual insects that feed on plants with higher levels of these compounds may suffer fitness consequences (Camara, 1997; Fordyce and Nice, 2008; Smilanich et al., 2009). Yet, at the same time, sequestered compounds may be important in protecting insects from their own enemies, and higher levels of these compounds may provide enhanced protection (Skelhorn and Rowe, 2006). While the efficacy of sequestered defensive compounds has been demonstrated for predators, less is known about how the compounds affect parasitoids.

There is little reason to assume that consumers at higher trophic levels are unable to evolve adaptations to dietary toxins, such as compounds sequestered by herbivores, yet relatively few studies have documented the transfer of sequestered compounds to higher trophic levels (e.g., McDougall et al., 1988; Rossini et al., 2000; Bowers, 2003; Reudler Talsma, 2007). Endoparasitoid larvae spend their entire larval development inside a single host where they often are exposed continuously to compounds sequestered by the host (Ode, 2006), and they are likely to ingest substantial amounts of these compounds. Because the mid- and hind-gut are not connected in hymenopteran endoparasitoids until emergence or pupation (Quicke, 1997), larvae of these species are incapable of egestion, and any ingested compounds not metabolized remain in their bodies. For instance, tomatidine, the aglycone of the alkaloid α-tomatine, was detected in larvae of the ichneumonid endoparasitoid, *Hyposoter exiguae,* that were developing within *Helicoverpa zea* caterpillars reared on alkaloid-spiked artificial diet (Campbell and Duffey, 1979). Several other studies have detected plant-produced or derived secondary metabolites in adult parasitoids, with concentrations magnified in adults of some species (McDougall et al., 1988; Campos et al., 1990; Reudler Talsma, 2007) and minute in others (Rossini et al., 2000; Bowers, 2003; Reudler Talsma, 2007).

In this study, we examined variation of catalpol and catalposide (a derivative of catalpol), two iridoid glycosides produced by Southern Catalpa trees, *Catalpa bignonioides* Walter (Bignoniaceae). We examined effects of these two defensive compounds on the primary herbivore of Catalpa, the Catalpa Sphinx, *Ceratomia catalpae* Boisduval (Lepidoptera: Sphingidae) and its primary endoparasitoid, *Cotesia congregata* Say (Hymenoptera: Braconidae). Iridoid glycosides (IGs) are monoterpene-derived secondary compounds that mediate interactions between plants, their herbivores and herbivore natural enemies (Bowers, 1993; Nishida, 2002 and references therein). Iridoid glycosides are sequestered by four orders of herbivores, including the Catalpa Sphinx (Bowers, 2003), and sequestered IGs can deter predators (e.g., Bowers, 1980; Bowers and Farley, 1990; Dyer and Bowers, 1996; Camara, 1997; Theodoratus and Bowers, 1999; Rayor and Munson, 2002). This study addressed three questions about variation in IGs in Catalpa trees, Catalpa Sphinx caterpillars, and *C. congregata* parasitoids: 1) Does IG content vary within and among populations of Catalpa trees, and is this variation associated with the presence of Catalpa Sphinx larvae? 2) Is the IG content of Catalpa Sphinx caterpillars correlated with the IG content of the trees on which they fed? 3) Are larvae of the parasitoid, *Cotesia congregata,* able to sequester IGs from their caterpillar hosts, and, if so, what is the relationship between IG concentrations in parasitoid larvae and caterpillar hosts?

Methods and Materials

Study System Catalpa bignonioides, the Southern Catalpa, is a widespread deciduous tree found throughout North America as an ornamental (Sibley, 2009). This species contains primarily two IGs, catalpol and catalposide, although a few others have been recorded (von Poser et al., 2000). The Catalpa Sphinx, *Ceratomia catalpae,* is a specialist on *Catalpa* spp. (Baerg, 1935; Nayar and Fraenkel, 1963; Bowers, 2003); larvae are gregarious and brightly colored, black, yellow and white, and sequester high concentrations of the IG catalpol from their host trees (Bowers, 2003). Adults are cryptically colored, grayish-brown; they do not contain IGs and are palatable to birds (Bowers and Farley, 1990).

A major natural enemy of Catalpa Sphinx larvae is the gregarious koinobiont parasitoid *Cotesia congregata*, which parasitizes larvae of a number of sphingid species, including Catalpa Sphinx (Harwood et al., 2002; Bowers, 2003). *Cotesia congregata* oviposit into 2nd-4th instar sphingid larvae and develop over ~2 wk, whereupon they emerge through the cuticle, spin silken cocoons, and pupate; adults emerge approximately 10 d later. Concentrations of catalpol in Catalpa Sphinx hemolymph, the sole source of nutrients for developing *C. congregata* larvae, can reach up to 50% dry weight (Bowers, 2003), and thus parasitoids developing in the hemocoel will be exposed to very high levels of IGs. Catalpol in the caterpillar host appears to have little negative impact on *C. congregata* fitness when reared from Catalpa Sphinx (Crocker, 2008; Lampert et al., 2010) despite evidence that catalpol is absorbed by developing parasitoid larvae (Bowers, 2003).

Leaf and Caterpillar Collections Leaves were collected 24–30 July 2008, from 14 separate *Catalpa bignonioides* stands at locations from New Jersey to Georgia (Table 1); trees in five of these stands were attacked by Catalpa Sphinx larvae. Trees in each population were sampled according to the same protocol. Approximately 10 leaves were removed from a single branch of caterpillar-free trees. To compare damaged and undamaged leaves in trees infested with Catalpa Sphinx larvae, approximately 10 leaves were collected from an undamaged branch and 10 from a damaged branch on each tree. At least 5 and up to 30 trees were sampled per site in this manner, depending on the number of trees in a stand. All leaves were dried for 2 d in an oven at 50°C, after which they were ground into fine powder using a coffee grinder.

In trees with larvae, all Catalpa Sphinx larvae within reach (~3.0 m) were removed. Larvae ranged from 2nd to 5th instar depending on the site. Larvae were stored alive in a plastic cooler until they could be processed in the laboratory in Boulder, CO, USA, where 5th instars (as determined by head capsule size) were immediately weighed to the nearest 0.1 mg and then frozen at −80°C for chemical analysis. Remaining larvae were used to collect parasitoids or to establish a laboratory colony.

Parasitoid Collections To determine the IG concentrations of parasitoid larvae and their caterpillar hosts, a separate set of Catalpa Sphinx larvae and larval *Cotesia congregata* were analyzed. Catalpol was measured in both caterpillar hemolymph and parasitoid larvae. For these data, Catalpa Sphinx larvae from a heavily parasitized population were collected in August 2008, from a mixture of catalpa trees at the USDA South Farm in Beltsville, MD, USA; the iridoid glycoside content of these trees was not measured. Tree species included *Catalpa ovata*, *C. x galleana* (a hybrid between *C. ovata* and *C. speciosa*), and *C. x erubescens* (hybrid of *C. ovata* x *C. bignonioides*). Twenty-four late 4th instars and 9 early 5th instars were included in chemical analyses. Larvae were anesthetized with CO_2, the abdominal horns were clipped, and 5 μl of hemolymph drained from these wounds were collected using microcapillary tubes and immediately placed in 5 ml methanol to extract catalpol. Caterpillars were dissected completely lengthwise and examined for *Cotesia congregata* larvae, which were removed, counted, washed with clean methanol, and dried with a paper towel. All members of a single *C. congregata* brood were pooled and weighed to the nearest 0.01 mg. *Cotesia congregata* larvae then were placed directly into methanol for catalpol extraction.

Tree Chemistry and Analyses A subsample of 25 mg of ground leaves was used for iridoid glycoside analysis. Sample preparation and analysis by gas chromatography (GC) followed previously described methods (Bowers and Stamp, 1993; Bowers, 2003). Briefly, samples were extracted overnight in methanol, filtered and evaporated to dryness, partitioned between water and ether to remove nonpolar compounds, and the water layer (containing the IGs) was evaporated. Phenyl-ß-D-glucopyranoside (PBG) was added as an internal standard. Standards composed of purified PBG, catalpol, and catalposide were used for instrument calibration. For GC analysis, iridoid glycosides were derivatized using Tri-sil Z™ (Pierce Chemical Company). GC analyses were performed on an Agilent 7890 system (Agilent Technologies) equipped with an Agilent DB-1 column (30 m, 0.320 mm, 0.25 μm particle size), and data were processed with Agilent ChemStations software (version B-03-01). Paired *t*-tests were used to compare catalpol and catalposide content between damaged and undamaged leaves of caterpillar-infested trees. A

Table 1 Locations from which *Catalpa bignonioides* leaves were collected, dates when leaves were collected during the summer of 2008, the number of trees sampled and the number of trees attacked by Catalpa Sphinx caterpillars in each population

Population	Latitude	Longitude	Date	Trees present	Trees attacked
Cecil Co., MD	39°41'38.76″	76°06′48.72″	25 July	6	4
Adams Co., PA	39°46′51.50″	77°15′37.63″	25 July	15	0
Frederick Co., MD	39°37′24.18″	77°25′19.38″	25 July	13	3
Franklin Co., GA	34°17′20.46″	83°05′14.82″	26 July	~25	0
Madison Co., GA	34°07′52.98″	83°12′57.60″	26 July	7	0
Clarke Co., GA	33°52′59.40″	83°25′12.06″	26 July	7	5
Johnston Co., NC	35°41′22.74″	78°23′55.80″	27 July	15	0
Botetourt Co., VA	37°37′11.04″	79°58′58.14″	27 July	8	3
Cumberland Co., VA	37°42′44.38″	78°10′43.14″	28 July	19	12
Caroline, Co., MD	38°42′27.78″	75°54′14.46″	29 July	7	0
New Castle Co., DE	39°24′20.22″	75°33′18.30″	29 July	15	0
Cumberland Co., NJ	39°23′58.50″	75°04′13.98″	29 July	8	0
Cape May Co., NJ	39°14′25.01″	74°49′44.56″	30 July	15	0
Ocean Co., NJ	40°00′38.64″	74°12′58.74″	30 July	12	0

random effects MANOVA was utilized to test for variation in catalpol and catalposide across populations, and profile analysis allowed for a comparison of variation of catapol vs. catalposide across populations. To examine the relationships between presence of caterpillars and leaf content of catalpol and catalposide in sampled trees, we utilized MANOVA and profile analysis, with population means of catalpol and catalposide as response variables and caterpillar presence as a fixed independent variable with three levels: trees from caterpillar-free populations, caterpillar-infested trees, and caterpillar-free trees from populations with caterpillars present. Residuals for both dependent variables were not significantly different from normal (Shapiro-Wilk test for normality, $P>0.3$ in either case). To avoid pseudoreplication, population means were utilized for these three levels of the independent variable. All linear contrasts between levels of independent variables were examined for the overall MANOVA model, and profile analysis tested the hypothesis that caterpillar presence affected catalpol differently from catalposide.

Caterpillar Chemical Analyses Fifth instar Catalpa Sphinx were removed from the freezer and immediately ground in methanol, with sand, to help macerate the insects. Iridoid glycoside extraction and analysis by GC were performed as described above. Fresh-dry weight conversions were obtained by weighing, drying, and re-weighing separate sets of larvae. The relationship between chemistry of leaves of individual trees and the catalpol sequestration by Catalpa Sphinx larvae was examined by using least-squares regression. Sequestration was compared among populations using ANOVA, with the percent dry mass of catalpol as the response variable. Because natural enemies such as vertebrate predators may consume an entire insect before finding it unpalatable, we also compared total catalpol amounts sequestered among populations by using analysis of covariance (ANCOVA), treating caterpillar dry weight as a continuous covariate and the quantity of catalpol (in milligrams) as the response variable. All statistical analyses were performed with SPSS and SAS 9.2.

Caterpillar Hemolymph and Parasitoid Chemical Analyses Capillary tubes of Catalpa Sphinx hemolymph were extracted for 72 h and *C. congregata* larvae were macerated with sand and extracted in 5 ml methanol for 48 h. Sample preparation and GC analysis were as described above. Because all *C. congregata* larvae from a single brood were ground and homogenized together, amounts of catalpol represented that absorbed by the entire parasitoid brood. Independent 2-tailed *t*-tests were performed to determine whether hemolymph catalpol content, parasitoid clutch size (number of larvae inside the host), parasitoid clutch dry mass, and parasitoid catalpol concentration (% dry mass) differed between 4th and 5th instar host larvae. Least-squares regression analyses were performed to determine if catalpol concentration in host hemolymph significantly influenced both the amount and concentration of catalpol in parasitoid larvae. Because all individuals from each brood were combined, we also performed multiple regressions to determine whether number and weight of parasitoid larvae and caterpillar catalpol sequestration influenced the amount and concentration of catalpol detected inside parasitoid larvae.

Results

Tree Chemistry Leaf iridoid glycoside content varied from <0.2 to 5.4% dry weight. There was no difference in the amount of catalpol ($t_{14}=1.44$, $P=0.17$), catalposide ($t_{14}=-0.33$, $P=0.74$), or total iridoid glycosides ($t_{14}=0.97$, $P=0.35$) between damaged and undamaged leaves within individual trees. Concentrations of both compounds were averaged for damaged and undamaged branches, and the mean was treated as the content of each tree for use in subsequent analyses. Catalpol and catalposide did, however, vary among populations (Wilk's $\lambda=0.5$, $F_{11,120}=10.10$, $P<0.001$), and profile analysis indicated that this variation was significantly greater for catalposide (Wilk's $\lambda=0.4$, $F_{11,120}=13.90$, $P<0.001$) (Fig. 1).

Leaf chemistry varied among trees in the three infestation categories (attacked trees, caterpillar-free trees from

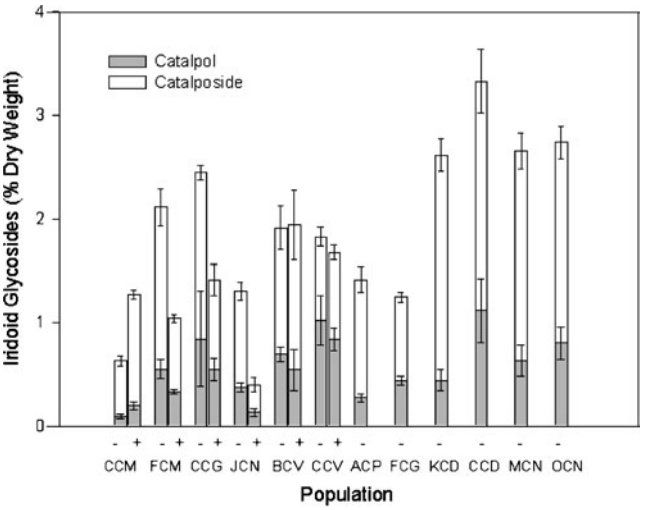


Fig. 1 Mean (± SEM) percent dry weight of catalpol and catalposide of all *Catalpa bignonioides* populations sampled. Plus signs (+) represent averages for trees attacked by Catalpa Sphinx caterpillars, while minus signs (−) represent averages for trees with no caterpillars. Abbreviations: **CCM** – Cecil Co., MD; **FCM** – Frederick Co., MD; **CCG** – Clarke Co., GA; **JCN** – Johnston Co., NC; **BCV** – Botetourt Co., VA; **CCV** – Cumberland Co., VA; **ACP** – Adams Co., PA; **FCG** – Franklin Co., GA; **KCD** – Kent Co., DE; **CCD** – Cumberland Co., NJ; **MCN** – Cape May Co., NJ; **OCN** – Ocean County, NJ

attacked populations, and trees from caterpillar-free populations) (Wilk's λ=0.7, $F_{2,15}$=3.60, P=0.05) (Fig. 2). Profile analysis showed that only catalposide concentration varied among the three infestation categories (Wilk's λ=0.6, $F_{2,15}$=4.60, P=0.03). According to pre-planned contrasts, leaves of trees from caterpillar-free populations had higher catalposide levels than trees with caterpillars (Tukey's P <0.05) (Fig. 2).

Caterpillar Chemistry As has been found in previous studies (Bowers, 2003; Lampert et al., 2010), only catalpol was detected in *C. catalpae* larvae. Percentage of catalpol sequestered by individual larvae ranged over an order of magnitude from 1.5-15.7% dry weight (Table 2), and was different among populations ($F_{4,73}$=13.45, P<0.001). After accounting for dry weight of larvae, the average mass of catalpol sequestered by individual larvae also varied significantly among populations ($F_{4,72}$=14.41, P<0.001) and many caterpillars contained over 15 mg of catalpol (Table 2). Furthermore, the percentage of catalpol in larvae was positively associated with the combined catalpol and catalposide concentrations in leaves upon which they fed ($F_{1,76}$=11.81, P<0.001) (Fig. 3). IGs sequestered by larvae increased with tree iridoid glycosides as expressed by the following equation: larva=1.61 * tree+5.22 (r^2=0.14).

Caterpillar Hemolymph and Parasitoid Chemistry Larvae from all *C. congregata* broods contained catalpol, although amounts varied substantially, ranging from 0.3-5.0% of their dry weight (mean=2.10±0.64). Neither the total mass of catalpol within parasitoids ($F_{1,29}$=0.003, P=0.95) nor percentage dry weight of catalpol ($F_{1,29\ =\ 0.31}$, P=0.58) inside parasitoid larvae was associated with hemolymph catalpol content of their host Catalpa Sphinx caterpillars.

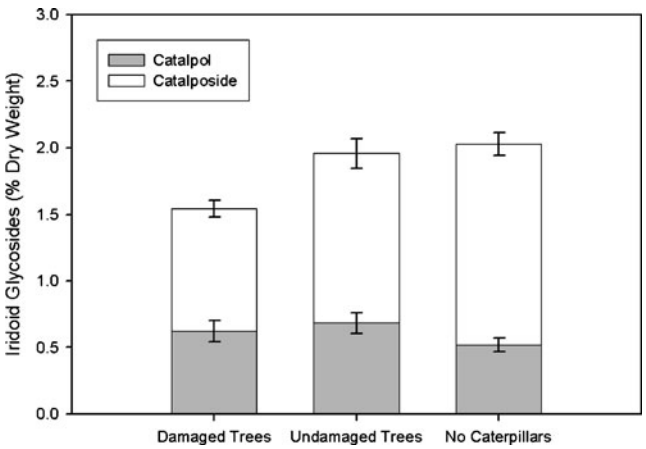


Fig. 2 Mean (± SEM) percent dry weight of catalpol and catalposide in *Catalpa bignonioides* trees attacked by Catalpa Sphinx caterpillars, caterpillar-free trees from attacked populations, and trees from caterpillar-free populations

Parasitoids dissected from 5th instars contained over twice as much catalpol (2.30% dry weight) as those dissected from 4th instars (1.18%) ($F_{1,28}$=7.48, P=0.01). According to the regression analysis, only total clutch size significantly (P=0.04) influenced parasitoid catalpol content. Larger clutches contained more catalpol (r^2=0.13, $F_{1,29}$=4.43, P=0.04).

Discussion

Variation in plant chemical defense is influenced by a combination of genetic and ecological factors, and the underlying genotypic and environmental influences on defensive chemistry can vary widely among plant populations. For *Catalpa bignonioides* leaves, there was considerable variation in IG content across the landscape in populations with and without caterpillars. This system is an attractive model study system due to this variation in defensive chemistry combined with the specialist feeding habits of associated caterpillars and parasitoids, and the variation in intensity of caterpillar and parasitoid attack among tree populations.

The presence of Catalpa Sphinx caterpillars was associated with quantitative differences in the IGs of *Catalpa bignonioides* leaves. In particular, catalposide concentration of trees with caterpillars was lower than that of trees with no caterpillars. However, caterpillar-damaged and undamaged leaves had similar catalpol and catalposide levels within individual trees, suggesting that there was no localized response to herbivory, or that sufficient time had elapsed to allow the response to become systemic or to attenuate. It may be that feeding by Catalpa Sphinx larvae suppresses IG production, as has been found for nicotine in wild tobacco in response to the specialist herbivore, *Manduca sexta* (Sphingidae) (Winz and Baldwin, 2001). If there is no effect of increasing levels of iridoid glycosides on this specialist caterpillar, then it may be more cost-effective for *Catalpa* trees to decrease production in response to herbivory to allow for greater allocation to other defenses; such a trade-off has been suggested for alkaloids vs. other defenses in *Senecio jacobaea* (Joshi and Vrieling, 2005). However, because we did not investigate female oviposition preferences, we have no way to determine whether the reduced IG concentration in trees with caterpillars was due to a direct effect of the caterpillar herbivory or whether females preferred trees that were lower in IGs. Whether or not feeding by Catalpa Sphinx larvae directly alters IG concentrations (induced resistance or induced susceptibility) requires further investigation.

Catalpa Sphinx larvae feeding on trees with leaves higher in IGs were themselves higher in IGs. This was found in a previous study (Lampert et al., 2010), but that

Table 2 Catalpol sequestration (range and mean±SEM) by 5th instar *Ceratomia catalpae* caterpillars from five populations

Population	# Larvae collected	Larval catalpol (mg)		Larval catalpol (%)	
		Range	Mean (± SEM)	Range	Mean (± SEM)
Cecil Co. MD	23	1.24–11.57	5.69±0.63 [b]	1.56–9.09	5.38±0.47 [b]
Frederick Co. MD	10	9.19–16.88	12.18±0.96 [a]	6.87–13.75	10.75±0.62 [a]
Clarke Co., GA	23	3.22–17.29	7.22±0.75 [b]	7.13–15.68	9.65±0.45 [a]
Botetourt CO., VA	23	9.62–9.01	12.81±2.16 [a]	1.56–15.68	8.27±3.67 [ab]
Cumberland Co., VA	23	5.84–23.60	12.19±0.83 [a]	3.85–13.56	7.57±0.58 [ab]

Lowercase letters within a column indicate significantly ($P<0.05$) different means according to Tukey's HSD test

study only considered six leaf samples. The present study examined IG content in over 160 individual trees and the caterpillars feeding on them. In this and previous studies, only catalpol was present in Catalpa Sphinx caterpillars (Bowers, 2003; Lampert et al., 2010). Although we have not done feeding trials where Catalpa Sphinx larvae are fed a diet with only catalposide, other studies with IG-sequestering species showed that esters of catalpol, such as catalposide, are hydrolyzed to catalpol and then sequestered (Gardner and Stermitz, 1988; L'Empereur and Stermitz, 1990). That is the most likely physiological process for Catalpa Sphinx larvae as well.

The positive correlation between caterpillar catalpol and iridoids of the leaves they were collected from has important ecological implications. No *Catalpa* tree species or cultivar has demonstrated resistance to Catalpa Sphinx (Olsen et al., 2006), regardless of secondary chemistry. Catalpol sequestration renders several caterpillar species unpalatable (Dyer and Bowers, 1996; Theodoratus and Bowers, 1999; Opitz et al., 2010), and Catalpa Sphinx larvae sequester high concentrations. Caterpillars that attack high-IG trees thus may be less palatable to their own natural enemies (with the exception of *Cotesia congregata*; Crocker, 2008; Lampert et al., 2010), and such trees may experience increased herbivory due to a combination of caterpillar phagostimulation and caterpillar escape from natural enemies.

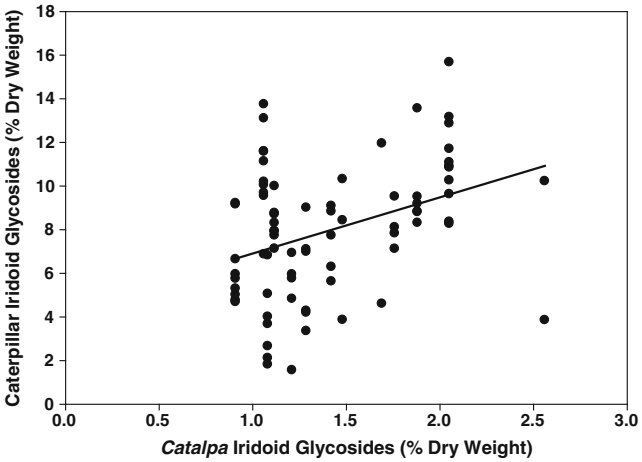


Fig. 3 Relationship between total iridoid glycoside content (catalpol plus catalposide) of leaves of individual *Catalpa* trees and the iridoid glycoside content (catalpol only) of the Catalpa Sphinx caterpillars collected from each tree

Parasitoid catalpol concentrations were much lower than concentrations in the hemolymph of host caterpillars. The fate of unsequestered catalpol absorbed by the parasitoid is unknown. Unaltered IGs, including catalpol, are water soluble and dissolved in caterpillar hemolymph (Bowers, 2003), the primary source of nutrition for *C. congregata*. Hymenopteran endoparasitoid larvae are unable to eliminate gut contents, and any ingested substance that is not metabolized by gut enzymes is expected to accumulate (Quicke, 1997). Indeed, in some systems, secondary compounds have been detected in higher concentrations in parasitoids than in the host (McDougall et al., 1988; Campos et al., 1990). However, our results suggest that relatively little catalpol is accumulated, while much is broken down. Also unknown is whether the detected catalpol is concentrated in the guts of the parasitoid larvae, or also is dissolved in their hemocoel. *Cotesia congregata* likely sequester some catapol in their hemocoel, as it has been detected in adults after meconium was eliminated (Bowers, 2003).

While parasitoids may sequester secondary compounds from their hosts (Benn et al., 1979; Fung, 1988; Rossini et al., 2000), it is still unknown whether parasitoid sequestration is an adaptive defense against natural enemies. Many parasitoids have a narrow host range, and those that attack sequestering hosts may be adapted to host chemistry. In this study, two hyperparasitoid genera, *Mesochorus* and *Hypopteromalus*, were reared from *C. congregata*, thus suggesting catalpol-rich *C. congregata* larvae are still susceptible to their own natural enemies. *Mesochorus* hyperparasitoids, which attack hosts in the larval stage (Baur and Yeargan, 1994), were reared from parasitoids collected in Beltsville, MD for the parasitoid chemistry analysis. A previous study (Lampert et al., 2010), as well as the present study, showed

no evidence that catalpol sequestration by either Catalpa Sphinx caterpillars or primary parasitoids deters *Mesochorus*. Reudler Talsma (2007) found that *Mesochorus* adults reared from *Cotesia melitaearum* [a specialist parasitoid of iridoid glycoside-sequestering *Melitaea* (Nymphalidae) caterpillars] also contained catalpol, presumably absorbed from their hosts. *Hypopteromalus* attack emerged *Cotesia* pupae (Gaines and Kok, 1999), and emerged from broods on the cuticle of caterpillars collected in Cecil Co., MD.

To our knowledge, no studies have directly examined the palatability of parasitoids that absorb secondary compounds from their hosts. *Cotesia congregata* reared from Catalpa Sphinx caterpillars appear to be palatable to predators, as ants have been observed carrying their pupae (Ness, 2003; Lampert, personal observation). Most likely these pupae contain catalpol concentrations too low to deter ants, although other studies have found catalpol to be highly deterrent to ants (e.g., Dyer and Bowers, 1996; Opitz et al., 2010).

Variation in chemical defense rarely has been described over multiple trophic levels. Secondary compound sequestration by herbivores is well known as an adaptive defense against natural enemies. However, predators and parasitoids often absorb and retain defensive compounds from herbivorous prey, and the ecological and adaptive consequences of defensive compounds present in the third trophic are unknown. Explicit tests of the palatability of carnivorous insects, particularly specialists like parasitoids, to their own natural enemies are required to determine if retention of defensive compounds is a physiological byproduct of predation or a potential adaptive response to natural enemies. Numerous multitrophic hypotheses assume that predators and parasitoids are less able to adapt to chemical defense than herbivores (e.g., Brower, 1958; Gauld et al., 1992), but this could be a misconception due to a dearth of studies that have examined various adaptations to chemical defense, such as catabolism or sequestration. More rigorous tests of multitrophic hypotheses about the ecology and evolution of plant-insect interactions will depend on a growing number of studies that have uncovered the effects of plant chemical defenses on predators and parasitoids.

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Introduction

In plants, research into the role of hybridization in the evolution of novel traits and new species is gaining momentum (Barton, 2001; Seehausen, 2004; Abbott et al., 2008). Hybrids have been used increasingly in experimental studies in ecology and evolution in part because interspecific hybrids (specifically segregating generations) often show greater variation in traits compared to parental species. Furthermore, segregating hybrids frequently show greater independence between different traits than the parental species (Hochwender et al., 2000; Lexer et al., 2003; Orians et al., 2010). Interspecific hybrids can have novel patterns of secondary chemical expression or accumulation compared to parental species, and sometimes can be more resistant or susceptible to herbivores than parental species (Rieseberg and Ellstrand, 1993; Fritz, 1999; Orians, 2000). This makes hybrids useful for studying the relationship between secondary metabolite variation and herbivores (Hallgren et al., 2003; Leiss et al., 2009).

Hybridization occurs frequently in the *Jacobaea* (syn. *Senecio*, Asteraceae) genus (Vincent, 1996). Members of this genus have been used extensively to study plant-herbivore interactions, which are largely mediated by a diverse group of pyrrolizidine alkaloids (PAs; see reviews by Hartmann, 1999; Macel, 2011). Twenty-six PAs have been reported from 24 *Jacobaea* species (Pelser et al., 2005). Pyrrolizidine alkaloids are ester alkaloids composed of a necine base (amino alcohol moiety) and an alkyl, or rarely aralkyl, necic acid (Hartmann, 1999). PAs can occur in two forms *in vivo*, the tertiary amine (free base) or the *N*-oxide form (Hartmann et al., 1989; Rizk, 1991; Wiedenfeld et al., 2008; Joosten et al., 2011). In *Jacobaea* species, all PAs except for senecivernine are derived from senecionine *N*-oxide, which is synthesized in the roots, transported to the shoots, and diversified into other PA structures (Hartmann and Toppel, 1987). Variation in PA structure and form can lead to variation in the performance of generalist insects and other plant enemies such as nematodes (van Dam et al., 1995; Macel et al., 2005; Dominguez et al., 2008; Thoden et al., 2009).

Jacobaea vulgaris (tansy ragwort or common ragwort, syn. *Senecio jacobaea*) is native to Europe and west Asia but invasive in North America, Australia and New Zealand. *Jacobaea aquatica* (marsh ragwort, syn. *Senecio aquaticus*) is closely related to, but not a sister species of, *J. vulgaris* (Pelser et al., 2003). Natural hybrids between these species occur in at least one location in The Netherlands (Kirk et al., 2004). The two parental species are attacked by different suites of specialist and generalist herbivores (personal observation). A previous study showed that artificial hybridization between these two species can be used to produce F_2 lines that are in some cases extremely susceptible, and in other cases extremely resistant, to generalist herbivores (Leiss et al., 2009).

Western flower thrips, *Frankliniella occidentalis* (hereafter WFT), is a key insect pest on a wide range of agricultural and horticultural crops globally (Kirk and Terry, 2003). Since this species is highly polyphagous and infests about 200 wild and cultivated host species (Yudin et al., 1986), *F. occidentalis* often is used as a representative generalist herbivore in studies of plant-insect interactions (e.g., Macel et al., 2005; Leiss et al., 2009). Previous studies have investigated the effects of PAs on WFT with experiments that used artificial diets (Macel et al., 2005), or demonstrated the relationship between PAs and WFT resistance in host plants (Macel, 2003; Leiss et al., 2009). These studies showed that PAs are toxic to WFT and play a role in the plant resistance against this insect. However, these authors incorporated only a limited number of PAs in their studies. Some PAs were not easily acquired for experiments. Only the major PAs were quantified in host plants, and PAs were measured without discrimination between the two forms due to technical limitations in analytical methods. The effects of PA variation in host plant on WFT resistance have not yet been tested.

The current study aimed to overcome the challenges associated with isolating many PA variants for diet studies by measuring WTF resistance in a segregating hybrid family, which is expected to demonstrate great variation in composition and concentration of secondary metabolites such as PAs. Additionally, technological advances now permit the detection of PAs that are present in extremely low concentrations or that demonstrate only slight structural variations compared to other PAs, thus allowing us to test the relationship between WFT resistance and PA composition using a comprehensive set of PAs *in vivo*.

We carried out WFT bioassays with an artificial hybrid family including one *J. vulgaris* genotype, one *J. aquatica* genotype, two F_1 offspring, and 94 different F_2 hybrid lines. We measured WFT feeding damage in the shoots of these genotypes, and investigated the relationship between PA variation and susceptibility to attack by WFT in the segregating F_2 generation. We addressed the following questions: 1) Is there variation in WFT resistance among segregating *Jacobaea* hybrids? 2) Is WFT resistance explained by PA concentration and composition? and if so, 3) Do different structural PA variants affect WFT resistance differently? 4) Are there any interactions between the effects of different PAs on WFT resistance?

Methods and Material

Study System and Plant Growth Jacobaea vulgaris seeds (collected at Meijendel Nature Reserve, 52° 7' 54" N, 4° 19'

46" E, The Netherlands) and *J. aquatica* seeds (collected at the Zwanenwater Reserve, 52° 48' 38" N, 4° 41' 7" E, The Netherlands) were germinated in glass vials. Clones were produced from tissue cultured seedlings, and several clones were subsequently grown in pots in climate rooms under standard conditions of 20°C, 70% relative humidity, light: dark 16 h: 8 h (Cheng et al., 2011). Potted plants were vernalized at 4°C with the standard light and humidity conditions for approximately 10 weeks to facilitate flowering. Both species are self-incompatible and crosses were performed by rubbing flower heads together (Kirk et al., 2005). Two rayed F_1 offspring were selected from this initial cross, and were reciprocally crossed with each other to produce two sets of F_2 offspring. One F_2 set consisted of 56 individuals and the other consisted of 46 individuals. The parental, F_1 and F_2 individuals were maintained in tissue culture and were cloned to perform experiments using replicate genotypes.

We grew about 6 cloned replicates per F_2 genotype and about 12 cloned replicates per parental and F_1 genotype for the WFT bioassay. In addition we grew the same number of replicates of the genotypes for PA analysis. The PA data for these genotypes were used both to study WFT resistance as described in this paper and for an analysis of patterns of PA profiles in *Jacobaea* hybrid plants that was published elsewhere (Cheng et al., 2011). The clones were individually potted in 1.3 l pots filled with 95% sandy soil (collected from Meijendel), 5% potting soil (Slingerland Potgrond company, Zoeterwoude, The Netherlands) and 1.5 g/l Osmocote slow release fertilizer (N:P:K=15:9:11, Scott®, Scotts Miracle-Gro, Marysville, OH, USA). Plants were kept in a climate room under standard conditions described above for 6 wk before the bioassay was initiated.

WFT Bioassay We used 12 replicates of each parental and F_1 genotype, and 3–6 replicates of each of 94 F_2 hybrid genotypes (6 replicates were used for most genotypes, though less than 6 were used in cases where plants died or were too small compared to other plants of the same genotype). A total of 587 plants were placed randomly in a climate room and grown under standard conditions. About 5870 adult WFT, previously reared on chrysanthemum (*Dendranthema grandiflora*), were released at evenly spaced points in the climate room. During the 3 wk feeding period, plants were watered every 2 d without wetting or disturbing the leaves. After 3 wk, silver damage caused by feeding from WFT on both upper and lower leaf surfaces was visually scored in mm^2 for each leaf, according to the methods developed by Leiss et al. (2009). Above ground plant parts (shoots) were harvested just above the root crown and dried for 3 d in an oven at 50°C before establishing the dry masses of the shoots.

PA Data Acquisition A Waters Acquity ultra performance liquid chromatographic system coupled to a Waters Quattro Premier XE tandem mass spectrometer (LC-MS/MS) (Waters, Milford, MA, USA) was used for PA analysis. Analysis was performed using a different set of the same tissue culture-derived clonal plants consisting of the same genotypes and number of clones as those used in the WFT bioassay, and grown under identical conditions. The plant shoots were harvested, stored at −80°C, and freeze-dried for 1 wk under vacuum with a collector temperature of −55°C. Dried plant material was ground to a fine powder and about 10 mg were extracted with 2% formic acid in a mass to volume ratio of 1:100. Heliotrine (Latoxan, Valence, France) was added as an internal standard to the extraction solvent at a concentration of 1 μg/ml. The extract was filtered through a 0.2 μm nylon membrane filter (Acrodisc, Pall Life Sciences, Ann Arbor, MI, USA). An aliquot (25 μl) of the filtered PA extract was diluted with water (975 μl) and injected into the LC-MS/MS system.

Seventeen individual PA standards were available for this study, representing the major PAs present in the plant extracts (Table 1). Senecionine, seneciphylline, retrorsine, and their corresponding *N*-oxides were obtained from Phytolab, Vestenbergsgreuth, Germany; senkirkine was obtained from Phytoplan, Heidelberg, Germany. Riddelliine and its *N*-oxide were obtained as authentic standards from Dr M. Chou (NCTR, Jefferson, AR, USA), and integerrimine was a gift of Dr. J. Trigo (UNICAMP, Campinas, Brasil). Jacobine and erucifoline were isolated from *J. vulgaris* plant material (PRISNA, Leiden, The Netherlands). The identity of the isolated standards was confirmed by ^{1}H-NMR and LC-MS analysis and by comparison with literature data (Logie et al., 1994). Acetylseneciphylline was obtained by acetylation of seneciphylline with acetic anhydride and pyridine, according to the procedure described by He et al. (2010). Integerrimine *N*-oxide, jacobine *N*-oxide, erucifoline *N*-oxide, and acetylseneciphylline *N*-oxide were prepared by *N*-oxidation of the corresponding tertiary amine PAs according to the procedure described by Christie et al. (1949) and adapted by Chou et al. (2003). The purity of the obtained standards was checked by LC-MS analysis and was at least 90%.

The other PAs listed in Table 1 were tentatively identified on the basis of their retention time, molecular mass and fragmentation pattern, and on comparison with PA standards and literature data. The presence of PA *N*-oxides was confirmed by selective reduction to the corresponding tertiary amines according to the method of Joosten et al. (2010). All PAs included in this study have been reported before as constituents of *J vulgaris* and/or *J aquatica* (Hartmann and Witte, 1995; Joosten et al., 2011; Langel et al., 2011) and no new PAs were identified.

Table 1 Pyrrolizidine alkaloids (PAs) detected in *Jacobaea aquatica*, *Jacobaea vulgaris* and hybrids

Group	PA	Retention time (min)	Precursor mass (m/z)	Fragment mass 1; 2 (m/z)	Collision energy 1; 2 (eV)	Standard PA used for quantification
Senecionine-like PAs (simple senecionine-related derivatives)	senecionine	9.93	336.2	94.0; 120.0	40; 30	senecionine
	senecionine *N*-oxide	6.97	352.2	94.0; 120.0	40; 30	senecionine *N*-oxide
	integerrimine	9.72	336.2	94.0; 120.0	40; 30	integerrimine
	integerrimine *N*-oxide	6.83	352.2	94.0; 120.0	40; 30	integerrimine *N*-oxide
	retrorsine	8.49	352.2	94.0; 120.0	40; 30	retrorsine
	retrorsine *N*-oxide	6.01	368.2	94.0; 120.0	40; 30	retrorsine *N*-oxide
	usaramine	8.29	352.2	94.0; 120.0	40; 30	retrorsine
	usaramine *N*-oxide	5.89	368.2	94.0; 120.0	40; 30	retrorsine *N*-oxide
	riddelliine	7.91	350.2	94.0; 138.0	40; 30	riddelliine
	riddelliine *N*-oxide	5.48	366.2	94.0; 118.0	40; 30	riddelliine *N*-oxide
	seneciphylline	9.16	334.2	94.0; 120.0	40; 30	seneciphylline
	seneciphylline *N*-oxide	6.36	350.2	94.0; 138.0	40; 30	seneciphylline *N*-oxide
	spartioidine	8.96	334.2	120.0; 138.0	30; 30	seneciphylline
	spartioidine *N*-oxide	6.36	350.2	94.0; 138.0	40; 30	seneciphylline *N*-oxide
	acetylseneciphylline	11.80	376.2	120.0; 138.0	30; 30	acetylseneciphylline
	acetylseneciphylline *N*-oxide	8.86	392.2	94.0; 118.0	40; 30	acetylseneciphylline *N*-oxide
	senecivernine	10.09	336.2	94.0; 120.0	40; 30	integerrimine
Jacobine-like PAs (jacobine-related derivatives)	jacobine	7.89	352.2	120.0; 155.0	30; 30	jacobine
	jacobine *N*-oxide	5.49	368.2	120.0; 296.0	30; 25	jacobine *N*-oxide
	jacoline	6.13	370.2	94.0; 138.0	40; 30	jacobine
	jacoline *N*-oxide	4.39	386.2	94.0; 120.0	40; 30	jacobine *N*-oxide
	jaconine	8.75	388.2	94.0; 120.0	40; 30	jacobine
	jaconine *N*-oxide	5.77	404.2	94.0; 138.0	40; 30	jacobine *N*-oxide
	jacozine	7.23	350.2	94.0; 138.0	40; 30	jacobine
	jacozine *N*-oxide	5.11	366.2	94.0; 118.0	40; 30	jacobine *N*-oxide
	dehydrojaconine	7.86	386.2	94.0; 120.0	40; 30	jacobine
Erucifoline-like PAs (erucifoline-related derivatives)	erucifoline	7.56	350.2	94.0; 120.0	40; 30	erucifoline
	erucifoline *N*-oxide	4.80	366.2	94.0; 118.0	40; 30	erucifoline *N*-oxide
	acetylerucifoline	10.18	392.2	94.0; 118.0	40; 30	erucifoline
	acetylerucifoline *N*-oxide	7.17	408.2	94.0; 120.0	40; 30	erucifoline *N*-oxide
Otosenine-like PAs (otosenine-related derivatives)	senkirkine	7.31	366.2	122.0; 168.0	30; 25	senkirkine
	otosenine	5.60	382.2	122.0; 168.0	30; 25	senkirkine
	onetine	4.35	400.2	122.0; 168.0	30; 30	senkirkine
	desacetyldoronine	6.26	418.2	122.0; 168.0	30; 30	senkirkine
	florosenine	8.35	424.2	122.0; 168.0	35; 30	senkirkine
	floridanine	6.79	442.2	122.0; 168.0	30; 30	senkirkine
	doronine	9.01	460.2	122.0; 168.0	30; 30	senkirkine

Data were recorded in multiple monitoring mode (MRM) using two selected precursor ions to product ion transitions per compound. The MS settings are shown in Table 1. For quantification, the sum of the two peak areas obtained for each compound was normalized against the peak area of the internal standard. Quantification was performed against a standard solution (100 μg/l) of the PAs in an extraction of tansy (*Tanacetum vulgare*), a plant known to be free of PAs. The use of a PA standard solution in blank plant extract was considered to be a more reliable approach than quantification against a PA solution in solvent only. This PA standard extraction was injected into every 30 samples and the averaged response was used for quantification. For those PAs without standards available, a semi quantitative (indicative) value was obtained by comparison with the most closely related analogue (e.g., an isomer) as indicated in Table 1. Data processing was conducted with Masslynx 4.1 software (Waters, Milford, MA, USA).

PA expression is genetically controlled under standard growth conditions, and PA production is not induced in shoots by aboveground herbivory in *Jacobaea* plants (Vrieling and Bruin, 1987; van Dam et al., 1993; Vrieling et al., 1993). Therefore, we averaged the concentration of

each PA across all clones of each genotype and used the genotypic mean concentrations in the analyses presented here. The 37 PAs identified from the *Jacobaea* hybrids could be classified into four types, according to their structural characteristics, biosynthetic pathways, and expression pattern: senecionine-like PAs, jacobine-like PAs, erucifoline-like Pas, and otonecine-like PAs (Pelser et al., 2005; Cheng et al, 2011). We followed this classification in this study (Tables 1–2). The total PA concentration as well as the amount for each structural group was calculated by summing the concentrations of the individual PAs.

Data Analysis We used general linear models to determine whether WFT resistance differed according to plant genotype. Feeding damage (dependent variable) was log-transformed to achieve normality, and plant genotype was defined as the independent variable with plant dry mass as covariate. Normal distributions and homogenous variances were confirmed by testing the residuals of the models using Shapiro tests and Bartlett tests respectively. Two models were set up: in the first, *J. vulgaris* was used as a reference, and in the second, *J. aquatica* was used. All other genotypes were compared to the reference in the model.

Table 2 Pearson / Spearman correlation tests between western flower thrips (WFT) feeding damage and the concentrations of individual pyrrolizidine alkaloids (PAs) in the 94 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris*

Group	PA	r/r_s	P	Adjusted P[b]
Senecionine-like PAs	senecionine	−0.247	*	ns
	senecionine *N*-oxide	−0.247	*	ns
	integerrimine	−0.292	**	ns
	integerrimine *N*-oxide	−0.243	*	ns
	retrorsine	−0.201	+	ns
	retrorsine *N*-oxide	−0.104	ns	ns
	usaramine	−0.013	ns	ns
	usaramine *N*-oxide [a]	0.11	ns	ns
	riddelliine [a]	0.217	*	ns
	riddelliine *N*-oxide	−0.109	ns	ns
	seneciphylline	−0.282	**	ns
	seneciphylline *N*-oxide	−0.214	*	ns
	spartioidine	−0.17	+	ns
	spartioidine *N*-oxide	−0.15	ns	ns
	acetylseneciphylline	0.044	ns	ns
	acetylseneciphylline *N*-oxide	0.062	ns	ns
	senecivernine	−0.323	**	+
Jacobine-like PAs	jacobine	−0.281	**	ns
	jacobine *N*-oxide	−0.322	**	+
	jacoline	−0.296	**	ns
	jacoline *N*-oxide	−0.331	**	*
	jaconine	−0.278	**	ns
	jaconine *N*-oxide	−0.325	**	*
	jacozine	−0.141	ns	ns
	jacozine *N*-oxide	−0.057	ns	ns
	dehydrojaconine	−0.102	ns	ns
Erucifoline–like PAs	erucifoline	−0.113	ns	ns
	erucifoline *N*-oxide	−0.081	ns	ns
	acetylerucifoline	−0.209	*	ns
	acetylerucifoline *N*-oxide	−0.195	+	ns
Otosenine–like PAs	senkirkine [a]	0.088	ns	ns
	otosenine	−0.056	ns	ns
	onetine	−0.086	ns	ns
	desacetyldoronine	−0.019	ns	ns
	florosenine [a]	0.252	*	ns
	floridanine [a]	0.234	*	ns
	doronine [a]	0.218	*	ns

[a]PAs with concentrations that were not normally distributed, for which Spearman correlation tests were carried out, while Pearson correlation tests were carried out for all other PAs

[b]*P*-values of the correlation testes were adjusted by Bonferroni method

Significance codes: [ns]not significant, [+] $P<0.1$, [*] $P<0.05$, [**] $P<0.01$

Differences between the hybrid and parental genotypes were evaluated by using the regression coefficient matrices of the two models. In each matrix, the estimated coefficient of a hybrid indicated whether it had suffered more or less damage than the reference genotype, and the *P* value showed whether the difference was significant (Crawley, 2005). This is similar to a *post-hoc* test of an ANOVA model, however, such a *post-hoc* test includes all pair-wise comparisons between groups, and we were interested only in testing for differences between hybrid and parental genotypes. The difference between the two parental genotypes was also tested by using the same regression coefficient matrices. WFT resistance of each hybrid genotype was categorized according to these definitions: ND—no difference, leaf damage area of the hybrid was not different from that of both parents; A—additive, damage was intermediate between that of the parents; Ds—susceptible-dominant, damage was similar to that of the susceptible parent; Dr—resistant-dominant, damage was similar to that of the resistant parent, S—susceptible, damage was greater than that of the susceptible parent; R—resistant, damage was less than that of the resistant parent (Supplementary Table 1).

For correlation tests and principal components analysis, we included only data from F_2 genotypes, since we were interested in using the variation from this segregating generation to search for underlying relationships between WTF resistance and PA expression. We were not able to test for differences in these relationships between the different generations described in this study because only a limited number of genotypes were included from the parental and F_1 generations. However, the parental and F_1 plants provided reference points for WFT resistance comparison. We used log-transformed genotypic mean values of feeding damage and PA concentrations to carry out correlation analyses. Either Spearman (for six minor PAs that did not have normally distributed concentrations) or Pearson correlation tests were carried out to test the relationship between feeding damage and the concentrations of individual PAs, pooled concentrations of each of the four PA groups and total PA (see details in Table 2 and Fig. 3).

Pyrrolizidine alkaloids from within structural groups were closely correlated with each other, and it was not possible, therefore, to investigate the interactions between them. The PAs from different structural groups, however, were generally expressed independently. The sum concentrations of the PAs from the four groups were not correlated with one another (Cheng et al., 2011). We used a multiple-regression model to test for interactions between the effects of different PA structural classes on feeding damage. In this model, feeding damage (represented by log-transformed genotypic mean values) was defined as the dependent variable, and the sum concentrations of each of the four PA structural groups (log-transformed and centered genotypic mean concentrations) were defined as independent variables.

The principal component analysis (PCA) was carried out by using log-transformed genotypic mean concentrations of all individual PAs except the six minor PAs that did not have normally distributed concentrations. Compared to the major PAs, these six PAs were present at very low concentrations (on average less than 1% of total PA concentration). Pearson correlation tests were carried out between the first six principle components (PCs) from the PCA and feeding damage. In order to evaluate the contribution of each PA to each PC (in other words the loading), Pearson correlation tests were carried out between individual PAs and the first 3 PCs, since PCs four to six

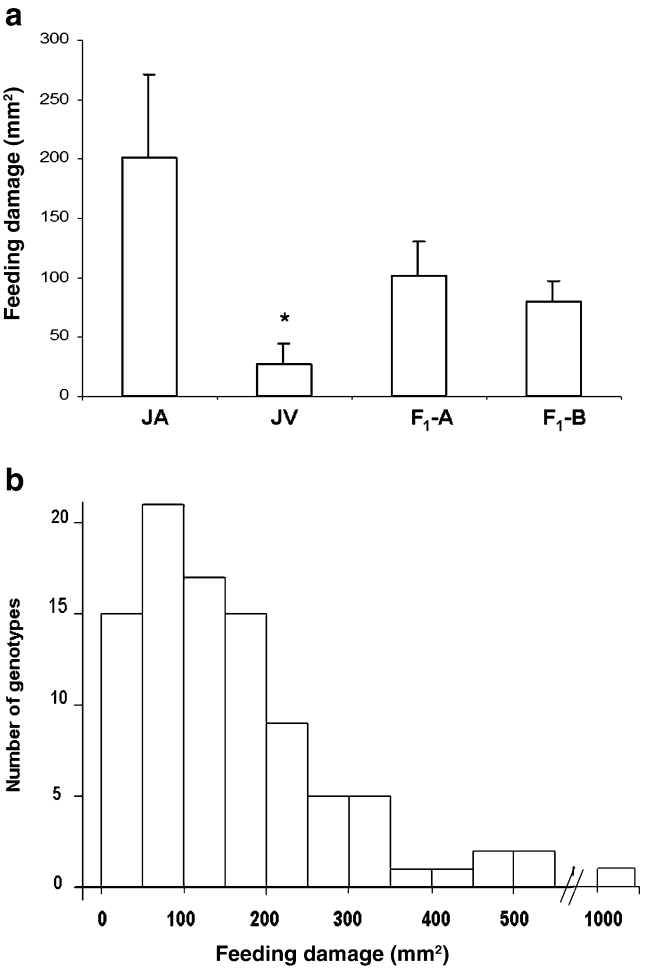


Fig. 1 Variation in western flower thrips (WFT) feeding damage (mm^2) on *Jacobaea aquatica, Jacobaea vulgaris*, 2 F_1 and 94 F_2 hybrids. **a** Mean feeding damage for one *J. aquatica* genotype (JA), one *J. vulgaris* genotype (JV), and 2 F_1 (F_1-A and F_1 -B) genotypes. Error bars are standard errors, $N=12$. *J. vulgaris* was significantly different from the other genotypes at * $P<0.05$. **b** Distribution frequency for genotypic mean WFT feeding damage of 94 F_2 hybrids. $N=3$–6 for each genotype. In total, 587 plants were used in WFT bioassay

accounted for a low proportion of the total variation and were not correlated with WFT feeding damage. The *P*-values were adjusted using the sequential Bonferroni method when multiple tests were carried out.

All analyses were conducted in R version 2.10.0 (R Development Core Team, 2009)

Results

Variation in Feeding Damage Feeding damage was genotype-dependent (*d.f.*=97,488; F=5.30; P<0.001). Plant mass also had effect on feeding damage (*d.f.*=1,488; F=18.44; P<0.001). Among the two parental genotypes, *J. aquatica* suffered more feeding damage than *J. vulgaris* (*d.f.*=1, 22; t=6.18; P<0.001). Both of the F_1 lines were as susceptible as *J. aquatica*. Among the 94 F_2 hybrids, 69 were as susceptible as *J. aquatica*, 10 were more susceptible than *J. aquatica*, 15 showed intermediate resistance, 9 were as resistant as *J. vulgaris*, and none were more resistant than *J. vulgaris* (Fig. 1, see statistical details in Supplementary Table 1).

Relationship Between Feeding Damage and PA Concentration Correlation tests between feeding damage and individual PAs showed that feeding damage was negatively correlated with the concentrations of the *N*-oxides of two jacobine-like PAs (jaconine and jacoline). Jacobine *N*-oxide concentration was marginally correlated with feeding damage, and the correlations between the free bases of jacobine-like PAs and feeding damage were not significant after correction for multiple testing. No other individual PAs were correlated with feeding damage (Table 2). Total PA concentration also was correlated with feeding damage (Fig. 2a). Of the four structural groups of PAs, only the sum concentration of jacobine-like PAs was significantly correlated with feeding damage by WFT (Fig. 2c). The sum concentrations of the other three groups were not correlated with feeding damage (see the statistical results for senecionine-and erucifoline-like PAs in Fig. 2b,d; for otosenine-like PAs: *d.f.*=92, r=0.35, P=0.77).

The multiple regression models showed that among the four PA groups only jacobine-like PAs had significant negative effects on feeding. There were no two-way interactions between the groups. A three-way interaction between senecionine-like, jacobine-like and erucifoline-like PAs and an interaction between the four PA groups were present. However, these were only marginally significant (0.05<P< 0.1, Table 3).

Relationships Between Feeding Damage and PA Composition We used principal component analysis (PCA) to reduce the PA data set to a smaller number of uncorrelated axes. PC1 explained 44%, PC2 explained 19% and PC3 explained 12% of the variation in the data. More than 90% of the total variation was accounted for by the first 6 PCs. Among first 6 PCs, PC1 was negative correlated (*d.f.*=92, r=−0.32, P=0.002) and PC3 was positively correlated with feeding damage (*d.f.*=92, r=−0.23, P=0.04). No other PCs

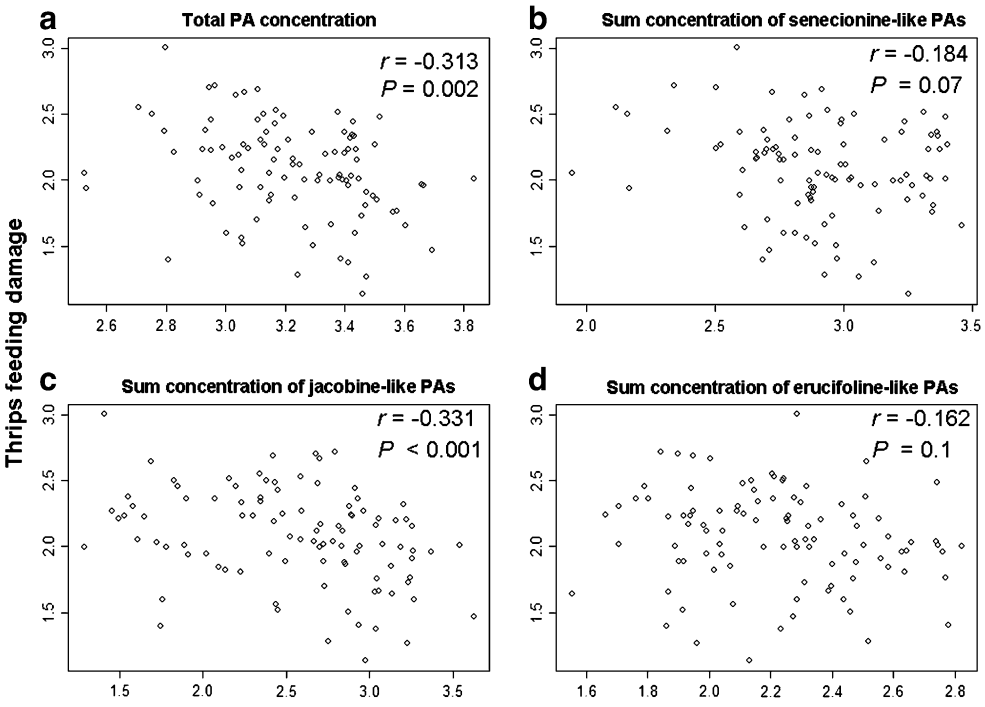


Fig. 2 Relationship between feeding damage by western flower thrips (WFT) (mm^2) and the concentration of total pyrrolizidine alkaloid (PA), senecionine-like, jacobine-like, and erucifoline-like PAs (μg/g dw) of F_2 hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris*. The data for WFT feeding damage and concentrations are the log-transformed genotypic mean values. In each panel, the results of the Pearson correlation tests between feeding damage and the PA concentrations are provided; in all cases, *d.f.*=92

Table 3 Results of multiple regression of western flower thrips (WFT) feeding damage (mm^2) against the sum concentration of four structural groups of pyrrolizidine alkaloids (PAs, μg/g dw) in the 94 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris* (For the regression model: adjusted R^2=0.1655; *d.f.*=15, 78; F=2.23; P=0.012)

	Predictors [a]	Estimate	*t* value
PA groups	snt	−0.22	−1.50
	jbt	−0.22	−2.83**
	ert	−0.10	−0.72
	onet	0.06	0.78
Two-way interactions	snt:jbt	−0.43	−1.45
	snt:ert	−0.26	−0.49
	jbt:ert	−0.27	−0.95
	snt:onet	0.05	0.21
	jbt:onet	0.15	0.93
	ert:onet	−0.03	−0.10
Three-way interactions	snt:jbt:ert	1.79	1.89 +
	snt:jbt:onet	0.43	1.07
	snt:ert:onet	−1.19	−1.29
	jbt:ert:onet	1.13	1.63
Four-way interaction	snt:jbt:ert:onet	−3.02	−1.71+

[a] snt, jbt, ert, onet: the sum concentration of senecionine-, jacobine-erucifoline- and otosenine- type PAs, separately

Significance codes: + P<0.1, * P <0.05, ** P<0.01

were correlated with feeding damage (data not shown). Correlation tests between each PC and individual PAs' concentrations allowed us to identify which PAs were associated with each PC. Jacobine-like PAs (except jacozine and its *N*-oxide) were strongly correlated with PC1, such that individuals with high PC1 scores had high concentrations of jacobine-like PAs. Variation in some senecionine-like, erucifoline-like PAs and otosenine-like PAs contributed strongly to PC3 (individuals with high PC3 scores had low/high concentrations of these PAs; Supplementary Table 2). A plot of PC1 vs. PC2 shows that F_2 hybrids can be divided roughly into different groups. Feeding damage, indicated by the size of the dots, is not clearly clustered either on the plot of PC1 vs. PC2 or the plot of PC_1 vs. PC_3 (Fig. 3).

Discussion

Segregating hybrids are sometimes used to study correlations and trade-offs between different ecologically important traits in plants, because they exhibit greater variation than parental species, and greater independence between traits (e.g., Orians et al., 2010). We showed that there is high variation in the WFT susceptibility among F_2 hybrids of *J. vulgaris* and *J. aquatica*. Although most F_2 hybrids were as susceptible as or even more susceptible than *J. aquatica* (73% and 11% among all F_2 hybrids respectively), there were still some hybrids with resistance similar to *J. vulgaris* (10%) or intermediate to the two parents (6%). The expression of PAs among the F_2 hybrid generation was highly variable (Cheng et al., 2011, but also see Fig. 2 and Fig. 3), and this variation provided an opportunity to investigate the *in vivo* effects of PA composition on plant resistance to a generalist herbivore.

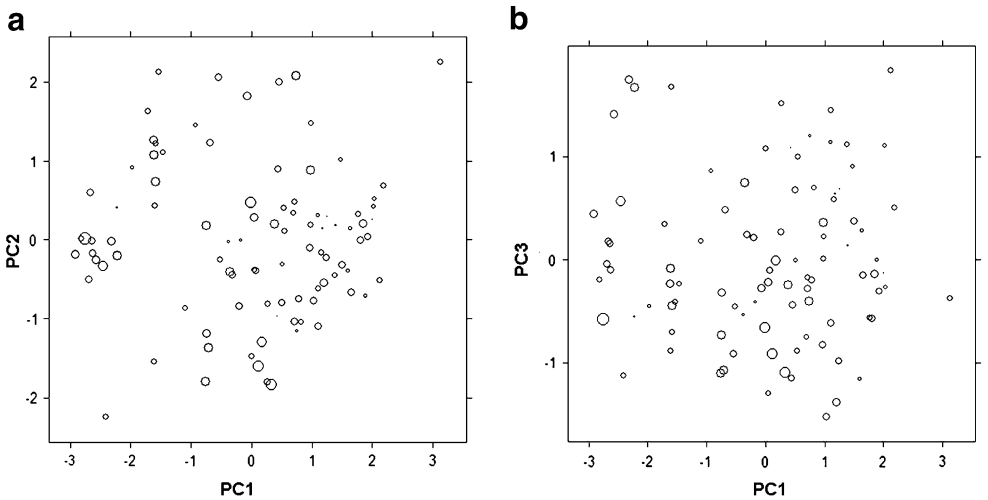


Fig. 3 Principal component analysis (PCA) of the pyrrolizidine alkaloid (PA) profiles of F_2 hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris*. PCA was performed on the log-transformed genotypic mean concentrations of all individual PAs excluding six minor PAs that did not have normally distributed concentrations (see Table 2). One dot represents one of 94 F_2 hybrid genotypes. Size of each dot represents mean WFT feeding damage for that genotype. The genotypic mean concentrations are the average value of the three to six replicates from the same genotype

We demonstrated that concentrations of total PA and jacobine-like PAs were negatively correlated with feeding damage by using correlation tests. The multiple regression and PCA also indicated that concentrations of jacobine-like PAs were more closely related to WFT resistance than concentrations of the other PAs. The important role of jacobine-like PAs in WFT resistance of *Jacobaea* plants also has been supported by previous studies. Macel (2003) found that WFT feeding damage was negatively correlated with total PA concentration and with jacobine (both *N*-oxide and free base) concentration in *J. vulgaris* plants. Leiss et al. (2009) found that resistant *Jacobaea* hybrids had higher concentrations of jacobine *N*-oxide and jaconine *N*-oxide than susceptible hybrids. To develop a better understanding of the deterrent effects of different PAs on WFT, bioassays should be conducted using pure samples of different PAs.

Macel et al. (2005) tested WFT larval survival on artificial diets containing six individual PAs including senecionine, seneciphylline, retrorsine, senkirkine, heliotrine, and monocrotaline, or mixtures of senecionine, seneciphylline, and retrorsine. The experiment indicated that toxic effects of PAs on WFT larva differed among the individual PAs. Furthermore, higher PA concentrations had more potent toxic effects, and no synergistic effects resulted from PA mixtures. These findings support the results of our study, with the caveat that our analysis revealed a potential weak interaction between the different kinds of PAs. However, the interactions were slight ($0.05<P<0.1$, Table 3), and it is difficult to interpret interactions between more than two predictors.

PA variation accounted for a relatively low proportion of the variation in feeding damage ($R^2=0.17$, Table 3). Therefore, other factors likely play roles in plant susceptibility to WFT. These factors may include plant physical characteristics such as plant size, which was found to be a significant covariate in this study. Total PA concentration and plant size together explained a slightly higher proportion of the total variation ($R^2=0.20$). Other secondary metabolites have been reported from these species and their hybrids, including flavonoids, kaempferol glucoside, and chlorogenic acid (Leiss et al., 2009; Kirk et al., 2011), and other phytochemicals such as sesquiterpene lactones may be present but remain unreported. These metabolites may also play a role in resistance to herbivores, individually or in interaction with PAs.

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studies, early stages (typically L1 or L2) of the host were attacked, whereby the hosts were a fraction of the size of the ovipositing female parasitoid. This means that the host had to grow considerably during the parasitism phase to achieve a size capable of supporting the minimal nutritional requirements of the parasitoid progeny. A commonly reported trend is that both hosts and parasitoids perform equally well (or poorly) on plants of a given quality, suggesting that the effects of plant-related traits on parasitoid performance are similar up the food chain (Barbosa et al., 1986; Gunasena et al., 1990; Ode et al., 2004; Harvey et al., 2005; Gols et al., 2008a,b; Morse, 2009; Lampert and Bowers 2010).

The effects of plant quality on the development of herbivores (and, by association their parasitoids), often has been invoked on the basis of *extrinsic* threats to survival. The 'slow-growth-high-mortality' hypothesis posits that the growth of hosts feeding on nutritionally poor diets will be prolonged, thus extending their 'window of susceptibility' to a suite of natural enemies such as predators and parasitoids as well as to abiotic stresses (Benrey and Dennor, 1997; Fordyce and Shapiro, 2003). Because the mortality of the parasitoid is proportional to that of the host during the interaction, parasitoids also will die in hosts that suffer precocious mortality as a result of pathogens of predators (Fritz, 1982). However, less attention has been paid to exploring the effects of plant quality on *intrinsic* (host-related) threats to the survival of parasitoids and to how these are temporally correlated. For instance, if the effects of ingested plant toxins are gradual and cumulative on the host, we might expect that parasitoids that terminate their relationship with the host earlier would survive better than parasitoids that emerge from older hosts. This would be especially true if host and parasitoid mortality are proportional on specific herbivore diets.

An important aspect of parasitoid biology that may be linked to differences in host plant quality is how the wasp regulates host growth relative to that of healthy hosts. This parameter is intimately correlated with the size of the adult parasitoid relative to the maximum potential size of its herbivore host. In many associations that involve macrolepidopteran hosts and their solitary koinobiont endoparasitoids, host growth is reduced by 90% or more, compared with healthy hosts (Jones and Lewis, 1971; Vinson, 1972; Smilowitz and Iwantsch, 1975; Harvey et al., 1999, 2004, 2010a). In other host-parasitoid associations, however, regulation of host growth is more subtle or absent entirely, at least in examples where the maximum size of healthy hosts and adult parasitoid size are not markedly different (Harvey et al., 1994; Gols and Harvey, 2009b). In situations where hosts pass through fewer instars and/or growth is arrested earlier in development, we might expect the development of solitary parasitoids to be less constrained by differences in plant quality than in situations where hosts must grow larger to satisfy the nutritional requirements of the parasitoid offspring. However, as far as we know, this assumption remains untested.

Here, we compared development of the cabbage moth, *Mamestra brassicae* L. (Lepidoptera: Noctuidae) and its solitary koinobiont endoparasitoid, *Microplitis mediator* Haliday (Hymenoptera: Braconidae) on wild cabbage plants originating from three populations growing naturally along the Dorset coast of Great Britain, as well as on two cultivars. Like other members of the large family Brassicaceae, cabbage plants produce inducible glucosinolates (hereafter GS) (Fahey et al., 2001). Glucosinolates and their breakdown products negatively affect the growth and/or development of herbivores (Li et al., 2000; Buskov et al., 2002; Agrawal and Kurashige, 2003; Hopkins et al., 2009). Previous work has shown that wild cabbages exhibit strong inter- and intrapopulation variation in heritable aliphatic GS (Giamoustaris and Mithen, 1995; Moyes et al., 2000; Gols et al., 2008a; Newton et al., 2009a,b). Cultivated cabbage is an important global crop. Due to the effects of artificial selection via domestication, plant breeders have often sought to reduce levels of foliar GS and to eliminate variation in various other plant traits (Gols and Harvey, 2009a). Despite this, cultivated strains of cabbage have been used extensively in ecological and behavioral studies (reviewed by Hopkins et al., 2009).

Here, survival, body mass and larval development time of *M. brassicae* and *Mi. mediator* were examined on three wild strains and two cultivars of *B. oleracea* that were either undamaged or induced by herbivore feeding. The three main aims of the current study were: (1) to compare the performance of an herbivore and its parasitoid on the various cabbage strains and to see if effects of plant quality on insect performance are similar in both organisms; (2) to determine if the effects of plant quality on development of male and female parasitoids are similar; and (3) to measure differences in GS concentrations in the three wild populations and two cultivars of *B. oleracea*, and by using these data, to determine if insect performance is correlated with qualitative or quantitative aspects of plant allelochemistry. We hypothesized: (1) that there would be a strong link between plant quality and both herbivore and parasitoid performance; (2) that this parameter would be correlated with total amounts of GS present in leaf tissues of *B. oleracea*; and (3) that previous herbivore damage would lead to increased levels of plant defense and that this also would be reflected in insect performance.

Methods and Materials

Plants Seeds of wild *B. oleracea* plants were collected from three populations growing in meadows adjacent to chalk cliffs along the south coast of Great Britain to the

west and north of Swanage, Dorset. The populations are located at sites known as 'Old Harry' (50°38′N, 1°55′E), 'Winspit' (50°35′N, 2°02′E), and 'Kimmeridge' (50°36′N, 2°07′E). In addition, two cultivated strains of *B. oleracea*: Brussels sprouts (var. gemmifera) cv. 'Cyrus', on which the lab culture of *M. brassicae* had been reared for many generations, and white cabbage (var. alba) cv. "Stonehead" were used. For simplicity, we also refer to the cultivars as 'populations'. Hereafter, the populations are abbreviated as OH (Old Harry), WIN (Winspit), KIM (Kimmeredge), CYR (Cyrus cultivar), and STH (Stonehead cultivar).

Seeds from the various populations were germinated in moist soil, and the seedlings were transferred to 2.1-l pots that had been filled with potting soil ('Lentse potgrond' no. 4, Lent, The Netherlands). Plants were grown in facilities operated by Unifarm at Wageningen University (WU), The Netherlands, in a greenhouse compartment at 18–25°C, 50–70% r.h. with a photoperiod of at least 16 hr. If the light intensity dropped below a level of 500 μmol photons m^2/s during the 16 hr photoperiod, supplementary illumination was supplied by high pressure mercury lamps. All plants were uniformly watered on a daily basis. When plants were 4 wk-old, they were fertilized once a week with Kristallon Blauw (16 N:6 P:20 K:3 Mg) at a concentration of 2.5 mg/l. Plants were 7 wk-old at the start of the experiments. Watering and fertilization continued during the experiments.

Insects Larvae of *M. brassicae* were collected originally from cabbage fields grown in the vicinity of Wageningen University, and had been reared for many years in the laboratory on Brussels sprout (*B. oleracea* var. gemmifera cv. Cyrus) plants. *Mamestra brassicae* is native to the Palearctic, considered to be a generalist herbivore, and is frequently a pest in cabbages and other crops. Newly emerged moths were placed in groups of 20 into plastic containers (35 by 20 cm) with 10% sugar solution absorbed into cotton wool in small plastic vials. Filter paper was placed into the containers as a cover, and the moths laid their eggs onto this substrate. Every day the filter paper was removed and stored at 7°C pending hatching. New filter paper was placed inside and over the containers. *Microplitis mediator* was collected from young *M. brassicae* caterpillars on potted cabbage plants that had been placed for a week into a flowering meadow adjacent to the Laboratory of Entomology at Wageningen University. This parasitoid also is native to Eurasia and develops in first (L1) to third (L3) instars of its hosts. It is a specialist of species of moths in the family Noctuidae. In Europe, *M. brassicae* is considered to be its most important host. Like other members of the higher Microgastrinae, larvae of *Mi. mediator* feed exclusively on host hemolymph, and thus consume only a small proportion of available host tissues. At maturity, the parasitoid larva perforates a hole in the side of the host and emerges to spin a brown, papery cocoon. The host dies within a few days.

Several hundred newly hatched L1 caterpillars were placed onto cabbage plants in a rearing cage (40×40×60 cm) and allowed to feed. After 24 hr, 60 wasps >5 d-old (about half of each sex) were released into the cage and allowed to parasitize the larvae for several days. Sugar solution was provided constantly, and new plants were supplied as necessary for the larval development. Larvae were allowed to develop until parasitoid egression and pupation. Cocoons of *Mi. mediator* were collected from cages and placed into large Petri dishes (15 cm dia.) until parasitoid eclosion.

Insects were reared in climate rooms at 23±2°C with a 16:8 hr L:D photoperiod.

Development of Mamestra brassicae and Microplitis mediator on Induced and Non-induced Brassica oleracea Leaves Healthy hosts: Egg batches of *M. brassicae* were allowed to hatch on the filter paper. Neonate larvae then were randomly allocated to 9.5-cm Petri dishes (7 larvae per dish) with excised middle-aged leaves from each of the 10 following plant treatments: induced or non-induced plants from the cultivars (CYR, STH) or from the wild populations (OH, KIM, WIN), with 10 dishes used per treatment. To induce *B. oleracea* plants, 10 L1 *Pieris rapae* larvae (distributed over 3 leaves) were allowed to move freely around and feed on the plants for 7 d after which time they were removed. Sections of leaf tissue from the herbivore-damaged or undamaged plants were provided as food for the caterpillars in the Petri dishes. Induced plants were prepared every 2 d.

A disc of moistened filter paper was inside each dish to prevent leaf tissues from desiccating. Every 2 d, leaf materials as well as the filter papers were replaced, and mortality of the larvae was recorded. Healthy larvae grow much larger than parasitized larvae. Therefore, healthy larvae from each Petri dish were transferred to larger plastic containers (15×10×10 cm) when they were L4. Mature larvae normally burrow into the soil in order to pupate; here, mature L5 larvae were provided with a layer of vermiculite approximately 3 cm deep in plastic boxes for this purpose. Pre-pupae that had stopped feeding and were in the vermiculite were carefully checked every day in order to determine when they had pupated. Fresh pupae were removed and weighed on an analytic balance (accuracy ± 0.1 mg). Larval development time was determined as the number of days between hatching and pupation.

Parasitized hosts: Newly molted L2 caterpillars that had been feeding on either non-induced or induced excised leaf tissues from the various cabbage populations were presented individually to mated female *Mi. mediator* wasps on

the end of a brush in small plastic vials. Parasitism was verified by a single insertion and removal of the ovipositor. Parasitized caterpillars then were placed into 9.5-cm Petri dishes (7 caterpillars per dish, 12 dishes per treatment) lined with moistened filter paper and provided with excised leaves from the same treatment group as they had been feeding on previously. Every 2 d, leaf materials as well as filter papers were replaced, and mortality of the larvae was recorded. Parasitoid larvae typically egress from the host and pupate after 8–9 d. Cocoons were transferred to and maintained individually in labeled glass vials. Newly emerged adult wasps were sexed, narcotized using $C0_2$, and weighed on a microbalance (accuracy 1 μg). Parasitoid eclosion was checked several times over the course of 24 hr. Development time (in hours) was determined as the duration between parasitism and adult emergence. All experiments were conducted in climate rooms at 23±2°C with a 16:8 hr L:D photoperiod.

Glucosinolate Analysis GS concentrations were measured in leaf tissues sampled from 8 to 10 plants from each treatment × population combination. Plants induced by *P. rapae* feeding were prepared similarly as in the developmental experiments. Non-induced and herbivore-induced plants were of the same age, and all plants were sampled at the same day. Two discs, 17-mm diam, were punched per leaf, one from each central part of a leaf half, and 6 fully unfolded leaves were sampled per plant. Leaf discs were pooled per plant and frozen in liquid nitrogen immediately after sampling and then stored at −80°C. Samples were later freeze-dried and pulverized. Glucosinolates were extracted using a methanol extraction, identified and quantified as described by van Dam et al. (2004). Briefly, GS were extracted with boiling 70% methanol solution, desulphatased with arylsulphatase on a DEAE-Sephadex A 25 column, and separated on a reversed phase C-18 column on HPLC with an acetonitril-water gradient. Detection was performed with a single wavelength detector set to 226 nm, with sinigrin used as an external standard.

Glucosinolates were classified based on their amino-acid origin into indole GS (derived from tryptophan), aromatic GS (derived from phenylanaline), and alipathic GS (derived from methionine). The latter group was further divided according to GS modification steps into sulfinyl, alkenyl, and hydroxylated GS (Halkier and Gershenzon, 2006).

Statistics To compare the effect of plant population and induction on the fitness correlates, development time, and body mass of both the herbivore and the parasitoid, we used an analysis of variance model with fixed (population and induction) and one random variable (Petri dish = replicate). In the analysis of the *Mi. mediatior* data, we also included sex as a fixed factor. The estimation of the effects in the model was based on restricted likelihood maximization (REML). The Satterthwaite method was used for approximating the denominator degrees of freedom for the *F*-tests of the fixed effects (Littell et al., 2002). To compare GS concentrations, we used a general linear model ANOVA with population and induction as factors. Development times were log transformed, and GS concentrations were log(x+1) transformed to meet assumptions of normality and equal variances. Data on the survival were analyzed by using non-parametric Kaplan-Meier survival analyses. The log-rank test was used to compare survival curves. Data were analyzed separately for the herbivore and the parasitoid. Parasitized hosts that did not produce a parasitoid were excluded from the analyses. All analyses were carried out using the statistical package SAS 9.1.

Multivariate statistics (PLS = Projection to Latent Structures) was used to reveal whether the variation in GS chemistry could explain the variation in the performance of the herbivore and the parasitoid. We used the PLS extension of the SIMCA P+ 12.0 software program (Umetrics AB, Umeå, Sweden) The program's cross validation procedure evaluates the significance of each additional component (starting with none) by comparing the goodness of fit (R^2) and the predictive value (Q^2) of the extended model with that of the reduced model. First, we ran the PLS analysis with all variables, and then we eliminated those GS variables from the model that contributed little to explaining the variation based on the variable importance on projection values (VIP values). Variables with VIP values >1 are most influential to the model (Eriksson et al., 2006). Per leaf sample, concentrations of the individual GS compounds, pooled levels for each of the GS classes as described in GS analysis section, served as variables in the model. Data were log transformed, mean-centered, and scaled to unit variance before they were subjected to analysis. Insect performance data, which represented the Y-matrix variables, included for the herbivore development time, pupal mass, and survival; and for the parasitoid for both sexes separately, development time, adult body size, and survival to cocoon formation (male and females combined).

Results

Development of Mamestra brassicae and Microplitis mediator on Induced and Non-induced Brassica oleracea Leaves Both development time ($F_{4,\ 73.7}$=270, P<0.001) and pupal mass ($F_{4,\ 92.3}$=100, P<0.001) of the herbivore *M. brassicae* were affected by the plant population on which the caterpillars had been feeding previously (Fig. 1a and b). Development time ($F_{1,\ 64.4}$=158, P<0.001) was longer and pupal mass ($F_{1,\ 75.3}$=52.1, P<0.001) was lower

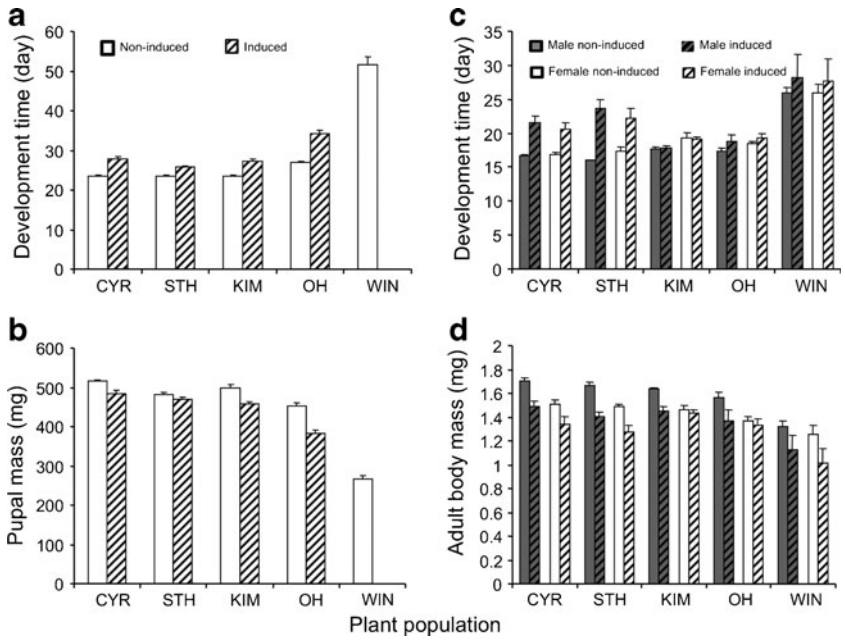


Fig. 1 Fitness correlates (development time and mass) of the herbivore *Mamestra brassicae* (**a**, **b**) and its parasitoid *Microplitis mediator* (**c**, **d**) when they were reared on cultivated (CYR or STH) or wild (KIM, OH, WIN) cabbage (*Brassica oleracea*). Hosts (healthy or parasitized) were reared in Petri dishes and provided with leaf tissues collected from non-induced plants (*open bars*) or from plants that had been exposed to *Pieris rapae* feeding for 7 d (*hatched bars*). Leaf tissues were replaced every other day. Parasitoids data are given for males (*grey* or *hatched grey bars*) and females (*white* or *hatched white bars*) separately. Bars are the mean values (+SE) based on pooled data per dish

on induced compared to non-induced plants (Fig. 1a and b). Induction differentially affected development time and pupal mass depending on the population on which the caterpillars had previously been feeding (population–induction interaction for development time $F_{3,\ 64.4}=5.68$, $P=0.002$, and for pupal mass $F_{3,\ 75.3}=5.12$, $P=0.003$). The effect of induction was strongest for WIN plants, as none of the larvae developed into pupae when they had been feeding on induced WIN plant tissues. Larval survival to pupation was high; more than 90% of the larvae successfully developed into pupae on all plant populations with the exception of the WIN population (Fig. 2a). Survival curves of larvae feeding on induced and non-induced leaf tissues were significantly different for the WIN population only (log-rank test, $P<0.001$).

Development time and biomass of the parasitoid on the various populations, exposed to previous feeding or not, reflected that of healthy hosts (Fig. 1). Plant population had an effect on development time of the wasps ($F_{4,\ 99.7}=39.4$, $P<0.001$); development time was longest on WIN leaf tissues. Moreover, the development time of the wasps was longer on induced than on non-induced plants ($F_{1,\ 110}=40.4$, $P<0.001$), and induction affected development differently depending on the plant population (population–induction interaction, $F_{4,\ 109}=8.80$, $P<0.001$). Previous herbivory tended to prolong development more on cultivated than on wild cabbage. The plant–sex interaction term also was significant ($F_{4,\ 331}=3.96$, $P=0.004$); females tended to develop slower than males on OH and KIM plants, while on the other populations this pattern was less clear. Plant population ($F_{4,\ 114}=19.9$, $P<0.001$), induction ($F_{1,\ 130}=38.9$, $P<0.001$) and sex ($F_{1,\ 478}=33.9$, $P<0.001$), all affected biomass of the adult wasps, with wasps being smaller on induced than on non-induced plants, and males being larger than females. Induction affected adult biomass of the males more strongly than that of the females (induction–sex interaction $F_{1,\ 478}=4.49$, $P=0.03$). Compared to healthy hosts, survival to pupation was more idiosyncratic for the parasitoid (Fig. 2b). Survival was higher on non-induced than on induced plant tissues for all populations except for the KIM populations (log rank tests: WIN, $P<0.001$; STH, $P<0.001$, OH, $P=0.003$, CYR, $P=0.02$, KIM, $P=0.95$). When survival curves on non-induced plant tissues were compared, it was similar on KIM, OH, and STH (log rank test was no longer significant after successive omission of CYR and WIN data). Using a similar approach, survival on induced plant tissues was not significantly different for OH, KIM and CYR plants.

Glucosinolate Analysis GS profiles differed profoundly for the different plant populations (Fig. 3). In CYR plants, the profile was dominated by sulfinyl GS (primarily 3-methylsulfinylpropyl GS), whereas in STH plants, it was dominated by alkenyl GS (primarily 2-propenyl GS). In the wild populations, there was both quantitative and qualitative variation in GS profiles. WIN plants produced high levels of alkenyl GS (especially 3-butenyl GS), and most

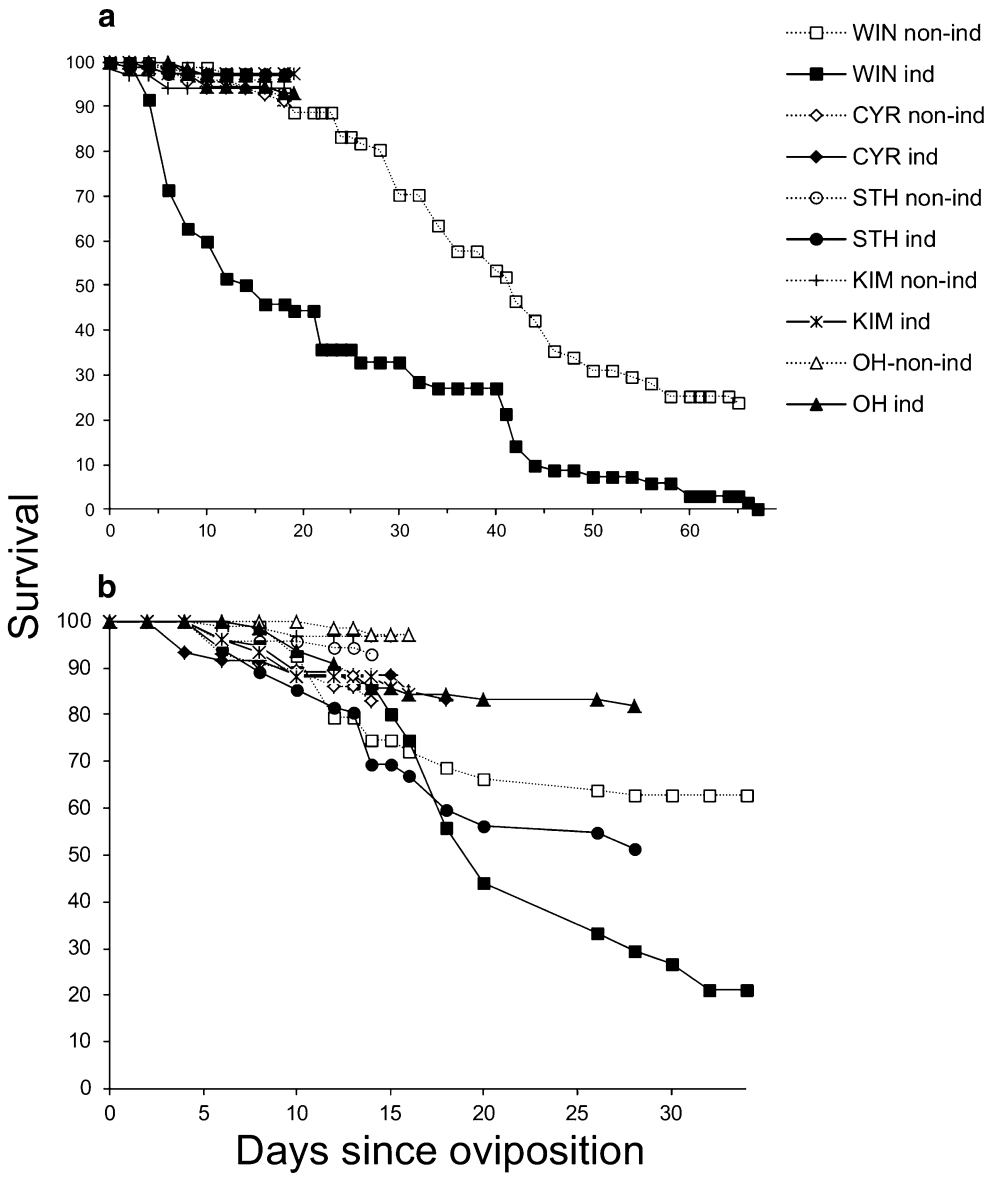


Fig. 2 Survival curves of healthy unparasitized *Mamestra brassicae* caterpillars (**a**) and caterpillars parasitized by *Microplitis mediator* (**b**). Survival was measured from hatching to pupation for healthy *M. brassicae* and for *Mi. mediator* from oviposition until cocoon formation. A few parasitized caterpillars developed into healthy caterpillars. These caterpillars were either not parasitized or the eggs were encapsulated and these were excluded from the data set

plants of this population contained small amounts of the aromatic GS 2-phenylethyl GS, whereas in the other two wild populations, all aliphatic GS classes were represented. Feeding by *P. rapae* increased levels of the indole GS and total GS concentration (indole GS, $F_{1,81}=59.0$, $P<0.001$; total GS, $F_{1,81}=10.0$, $P=0.001$). For the indole GS, the degree of induction depended also on the population (population–induction interaction $F_{4,81}=9.96$, $P<0.001$). The increase of indole GS was significant only for the wild populations (all P-values <0.001, Tukey-Kramer).

The final PLS analysis, from which the variables had been omitted that contributed little in explaining the variation, resulted in a model with two significant principal components explaining 46 and 25% (=R^2X), respectively, of the variance among the GS variables and 63 and 4% (=R^2Y), respectively, among the performance variables. The predictive value of this model was 65% (=Q^2). Each of the three performance parameters measured in healthy hosts, male wasps, and female wasps, respectively, were grouped together. Development time correlated negatively with both survival and biomass. Levels of 3-butenyl GS and 2-phenylethyl GS had a strong negative impact on insect performance, whereas levels of 3-methylsulfinylpropyl GS and 2-hydroxy-3-butenyl GS positively correlated with insect performance. Indole GS had little effect on insect performance based on PLS analysis and only separated the wild populations from the cultivars.

Discussion

The results of this study show that the development of *M. brassicae* and *Mi. mediator* was similar on the two cultivars

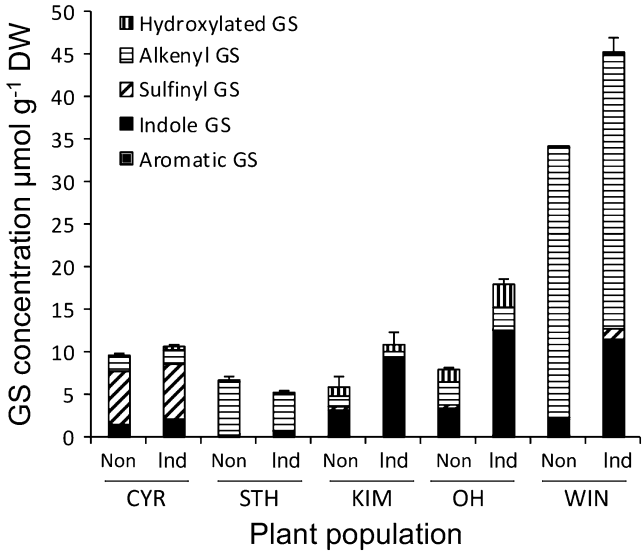


Fig. 3 Glucosinolate (GS) concentrations measured in samples taken from cultivated (CYR or STH) or wild cabbage (*Brassica oleracea*) plants originating from England (KIM, OH, WIN population). Samples were taken from non-induced plants (Non) and from plants that had been exposed to *Pieris rapae* feeding for 7 d (Ind). Concentrations of the detected 11 GS compounds were pooled for the three different GS classes: aromatic, indole, and aliphatic GS. Aliphatic GS were further subdivided into sulfinyl, alkenyl and hydroxylated GS. Bars give the mean values (+SE) for total GS concentrations. The number of samples varied between 8 and 10 per plant line and treatment

(CYR and STH) and the wild cabbage population KIM. Food plant quality of the wild populations ranked KIM>OH>WIN based on visual inspection of the data. There also was a general decline in fitness correlates of the host and parasitoid when developing on plants that had been previously induced by *P. rapae*. However, survival to pupation of healthy *M. brassicae* caterpillars was high on all but the WIN population, irrespective of whether the plants had been induced or not. When the caterpillars developed on WIN plants, survival decreased dramatically. Moreover, on induced tissues of the WIN population, all caterpillars died. Surprisingly, aside from the WIN population, the effects of induction on survival were more pronounced on the parasitoid, *M. mediator* than on the herbivore, *M. brassicae*. In contrast, on WIN plants, parasitoid survival was higher than survival of unparasitized caterpillars on both non-induced and induced plants.

In many plant–herbivore associations, early instars are more susceptible to plant allelochemicals and other plant quality characteristics than later instars. In this case, mortality spikes during the first instar and then levels off as the larvae grow and become better able to detoxify and/or excrete the plant's toxins (Zalucki et al., 2002). However, in our study, the effects of plant quality in WIN plants on the survival of *M. brassicae* and *Mi. mediator* were gradual on non-induced and induced plants. *Microplitis mediator* terminates its relationship with the host at an earlier stage of development (typically L3 or early L4), a point when the host is only approximately 5% of the mass of a fully grown healthy caterpillar (Fig. 4). We also noted that some caterpillars of *M. brassicae* feeding on WIN plants successfully molted to the 5th instar, but were quite small (<500 mg) and refused to eat after molting, generally perishing within a week or so. Furthermore, whereas the larval development of a healthy *M. brassicae* caterpillar can last up to 3 weeks or even longer, that of a parasitized caterpillar is less than half of this duration. This means that parasitized caterpillars are under fewer host-growth-related constraints than healthy caterpillars, and this may explain the discrepancy in survival of the host and parasitoid on induced and undamaged WIN leaf tissues, compared with the other strains/populations (Fig. 4). We have provided a conceptual graph (Fig. 4) showing that koinobiont parasitoids, such as *Mi. mediator*, which terminate their relationship with the host at a very early stage of development (e.g., when it is very small), may be less susceptible to plants of low quality than parastioids that either require the host to grow to a large final size or healthy hosts. This is because the effects of plant toxins may be cumulative over the course of herbivore/parasitoid development, or else because the insects are forced to interact with the plant for a longer period of time.

A previous study (Gols et al., 2008a) found that the performance of a specialist herbivore, *Pieris rapae*, and its solitary endoparasitoid *Cotesia rubecula* (a close relative of *Mi. mediator*) on the same wild populations of *B. oleracea* was less variable than found for the host and parasitoid used in this study. Moreover, whereas *M. brassicae* develops poorly on diets where concentrations of aliphatic and total GS are high, the specialist appears to be more sensitive to higher levels of indole GS (Gols et al., 2008a). This probably reflects adaptive differences in the two associations that relate to specialization via frequency-dependent selection. Using *Arabidopsis thaliana* plants with mutations that greatly reduced levels of aliphatic GS, indole GS, or both, Müller et al. (2010) demonstrated that the growth of *Trichoplusia ni*, *Manduca sexta*, and *Spodoptera exigua* caterpillars, three lepidopteran species, were all negatively affected by the presence of aliphatic GS. Only the growth of *S. exigua* was negatively affected also by the presence of indole GS (Müller et al., 2010). The effects of plant quality on development and survival of both generalist and specialist herbivores often are proportional up the food chain (Gunasena et al., 1990; Ode, 2006; Gols et al., 2008a,b; Harvey et al., 2011), or else less well-adapted parasitoids perform worse than their hosts as levels of direct plant defenses increase (Harvey et al., 2003, 2005; Ode, 2006; Gols et al., 2008b). By contrast, high concentrations of plant toxins sometimes impair the development

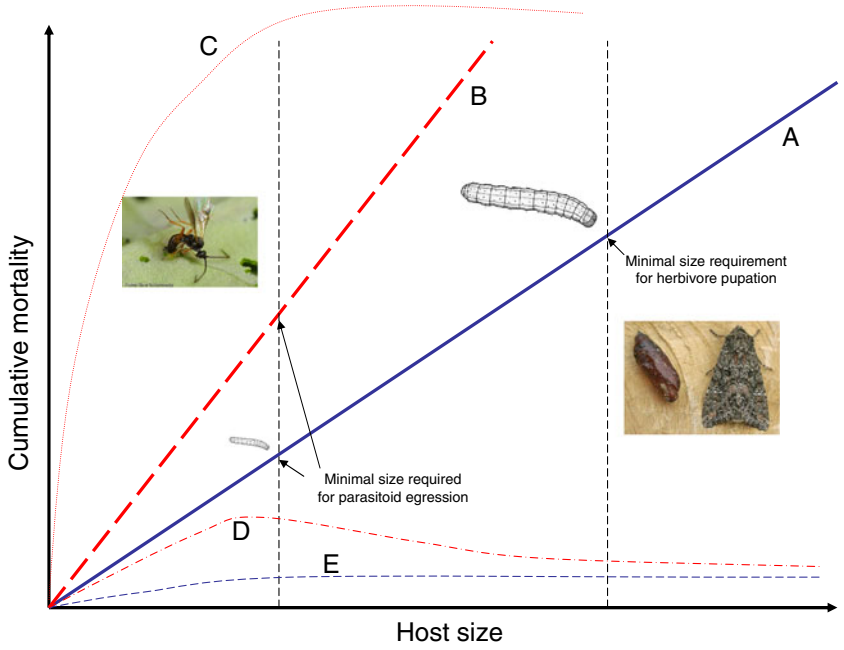


Fig. 4 Conceptual diagram showing the relationship between herbivore (healthy or parasitized) growth curves and cumulative mortality on food plants that differ in their quality. *Solid line* **a** and short *dashed line* **e** represent herbivore growth curves on non-induced plants; *long dashed line* **b**, *dotted line* **c** and *alternating dot-dashed line* **d** lines represent herbivore growth curves on induced plants. In this example, the parasitoid is solitary and the host caterpillar must reach a much smaller critical size to support parasitoid development (*left dashed black vertical line*) than to successfully pupate (*right vertical black dashed line*). In this study, induction steepens the mortality curve (lines **a**, **b**) reducing survival of both the parasitoid and the herbivore; the latter does not survive at all on induced plants because it cannot grow large enough to reach a minimum viable size for pupation. If the plant is even more toxic, then the parasitized host does not even reach a critical size and all parasitoids die before egression form the host (line **c**). In some instances, perhaps due to stress caused by parasitism, parasitoids may experience higher mortality on induced plants than their hosts (line **d**), or else herbivore and parasitoid mortality on non-induced plants does not differ (line **e**). Many different combinations are possible, depending on the effects of the plant on the herbivore and parasitoid, the parasitoid on the herbivore, and quantitative differences in the required growth of healthy and parasitized hosts

of the host more than that of the parasitoid (Devine et al., 2000; this study). In these examples, the outcome of the interaction may hinge on the effects that plant allelochemicals have on the physiology of the insects through processes associated with host-parasitoid nutritional ecology and/or on host immunity (Bukovinszky et al., 2009; Smilanich et al., 2009).

Analysis of allelochemicals in plant tissues revealed qualitative and quantitative variation among the populations. Furthermore, induction by *P. rapae* feeding mainly affected levels of indole GS, and these increased more in wild than in cultivated cabbage (see also Harvey et al., 2011). However, PLS analysis did not reveal a significant relationship between levels of indole GS and performance of the herbivore or the parasitoid. We only found a negative correlation between insect performance and levels of 3-butenyl GS and 2-phenylethyl GS. However, van Leur et al. (2008) demonstrated that the performance of *M. brassicae* was little affected when feeding on *Barbarea vulgaris* genotypes that produce 2-phenylethyl GS at much higher concentrations than the WIN plants used in this study. The effect of plant induction on survival was more pronounced in the parasitoid than in healthy hosts. This suggests that something other than GS, with perhaps the exception of 3-butenyl GS, is responsible for the decline in insect performance, especially for the parasitoid. Primary metabolites, such as nitrogen, phosphorous, and carbon, were not measured in the current study, but previous research with crucifers suggests that nitrogen, often a limiting nutrient for insect development, is always present in high concentrations in *Brassica* species (Soler et al., 2005). It is more likely that some unidentified secondary plant metabolite(s), that is/are present in leaf tissues of cabbage plants is induced after herbivore damage. For example, highly toxic cardenolides have been found in addition to GS in leaf tissues of the crucifer, *Erysimum cheiranthoides* (Renwick, 2002). Cipollini and Gruner (2007) have isolated cyanide from leaves of the garlic mustard, *Alliaria petiolata*. The invasive crucifer, *Bunias orientalis*, is highly toxic to specialist herbivores although its main allelochemical, the aromatic GS sinalbin, is also present in other crucifers that are suitable food plants for these herbivores (Harvey et al., 2010b).

In summary, this study has reported that differences in the quality of cultivated and wild cabbage plants affected the development and survival of a generalist herbivore and its solitary endoparasitoid. Differences in plant quality on insect performance were linearly correlated with concentrations of the aliphatic 3-butenyl GS, which in WIN plants exceeded some quantitative threshold for successful development. The poor quality of WIN plants also had a more serious affect on survival of the host, *M. brassicae*, than of its parasitoid, *Mi. mediator*, which probably reveals different temporal and physiological constraints imposed by the plant on both organisms. Thus far, more intimate aspects of life-history and development strategies in herbivores and their natural enemies in response to differences in plant quality have been largely ignored. Instead, researchers have tended to lump the insects according to their trophic status. By incorporating biological and physiological characteristics of the insects, such as their feeding strategies, growth patterns, and other relevant criteria, a better understanding of three (and higher) trophic level interactions will be attained.

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and Puterka, 2004). In addition to acyl sugars, nicotine, caffeoylputrescine, and several derivatives of the flavonoid, quercetin, are produced in trichomes and contribute to the leaf surface chemistry of *N. attenuata* (Roda et al., 2003). Many important steps in the biosynthesis of nicotine-related compounds take place in the trichomes. For example, nornicotine in *Nicotiana stocktonii* is converted in trichomes to *N*-hydroxyacylnornicotine, which subsequently is rapidly excreted onto the leaf surface (Zador and Jones, 1986). Such *N*-acylnornicotines (NacNN) have been shown to be a much more potent defense against the specialist herbivore *Manduca sexta* than nicotine itself (Jones et al., 1985). In *Nicotiana repanda,* the production of NacNN from nornicotine and its exudation to the leaf surface is rapidly elicited by methyl jasmonate (Laue et al., 2000). All these studies make inferences about trichome chemistry and function not by directly examining trichomes but rather by washing the leaf surface or even extracting complete leaf tissue.

In recent years, advances in non-destructive techniques such as NMR-spectroscopy have improved our ability to study intact tissues and plant organs. Hence NMR is the method of choice to gain a better understanding of the role of trichomes in the wild tobacco, *Nicotiana attenuata.* We examined wild type plants and a line of plants transformed with an RNAi construct harboring a fragment of the subunit of a SnRK1 (SNF1-related kinase) protein kinase (Schwachtje et al., 2006). This particular transformed line produced large trichomes with large basal cells (Supplemental Fig. S1E). Since only one of more than 20 transformed lines produced this phenotype, we infer that the trichome phenotype results from a t-DNA insertional mutagenesis event, rather than the RNAi of the SnRK1. These enlarged trichomes allowed us to harvest sufficient trichome mass to conduct NMR experiments and HPLC analysis to profile the secondary metabolites in the different cell types of type C trichomes and in the trichome heads of type D trichomes.

NMR spectroscopy and LC-TOF-MS analysis revealed for the first time the presence of phaseoloidin in the trichomes of a Solanaceous plant. This homogentisic glucoside was described previously from the seeds of *Entada phaseoloides* (Fabaceae) (Barua et al., 1988). Additionally, we showed in bioassays with artificial diets that phaseoloidin reduces larval growth of the specialist larvae *Manduca sexta* and the generalist larvae *Spodoptera littoralis,* suggesting a strong defensive function comparable to that of nicotine.

Methods and Materials

Plant Growth We used an isogenic line, obtained after 30 generations of inbreeding, of *Nicotiana attenuata* Torr. *ex* Watson originally derived from field-collected seeds. For trichome collection, a transformed line, (irGAL83; transformation construct described in Schwachtje et al. (2006)) containing a tDNA insertion mutation that resulted in the production of large trichomes. Seed germination was performed as described by Krügel et al. (2002). All plants were grown individually in 1 L pots in the glasshouse at 26–28°C under 16 hr of light supplied by Philips Sun-T Agro 400- or 600-W sodium lights.

Trichome Collection For the NMR studies, the two distal cells with the glandular vesicle of 20 trichomes (C tip, as indicated in Supplemental Fig. S1, panel A) or the following three to four cells (C middle) of flowering plants containing the insertional mutation were collected with tweezers. Approximately 150 heads of type D trichomes of flowering plants were collected with a glass capillary (D head). All samples were dissolved in 600 µl deuterated methanol directly after collection. Cells were sonicated before ^{1}H NMR measurements at 500 MHz using a cryogenic probe.

To estimate the amount of phaseoloidin and nicotine present in trichomes (Fig. 1), we harvested 60 type C trichomes from the midvein of rosette stage wild type *N. attenuata* leaves. Trichomes were excised with a razor blade and rinsed from the blade by dipping into a 4-ml vial containing 2 ml of methanol; a process that was repeated three times. Afterwards the samples were sonicated and the solvent was removed under a constant flow of nitrogen. The residue was then dissolved in 70 µl deuterated methanol, and 7.5 µg phloroglucinol (Fluka) were added as internal standard (ISTD) for quantification. Total amounts of phaseoloidin and nicotine were calculated according to formula 1–2, described in Fig. 1.

Isolation of Phaseoloidin Seeds of *Entada phaseoloides* (90 g) were obtained from Urban Jungle (Munich, Germany). The seeds were ground and defatted with *n*-hexane (200 ml each) × 3 and extracted with 80% aqueous ethanol (300 ml) × 3. The alcoholic extract was concentrated, and subsequently fractionated with *n*-hexane, ethyl acetate, *n*-butanol/water. The ethyl acetate fraction (5.0 g) was subjected to two silica gel 60 (40–63 µm, *Merck*, Darmstadt, Germany) column chromatographic separations and eluted with *n*-hexane/ethyl acetate (9:1, 8:2, 7:3, 6:4). The fractions were monitored by TLC on silica gel 60 F_{254} plates (*Merck*), using a mixture of n-hexane/ethyl acetate 13:7, and visualized under a UV lamp (254 nm) after heating and spraying with phosphomolybdic acid in ethanol. The similar fractions were combined. Ten fractions (200.0 mg) contained crude phaseoloidin and were subjected to a second column chromatography separation to produce 150.0 mg semi-pure phaseoloidin, which was

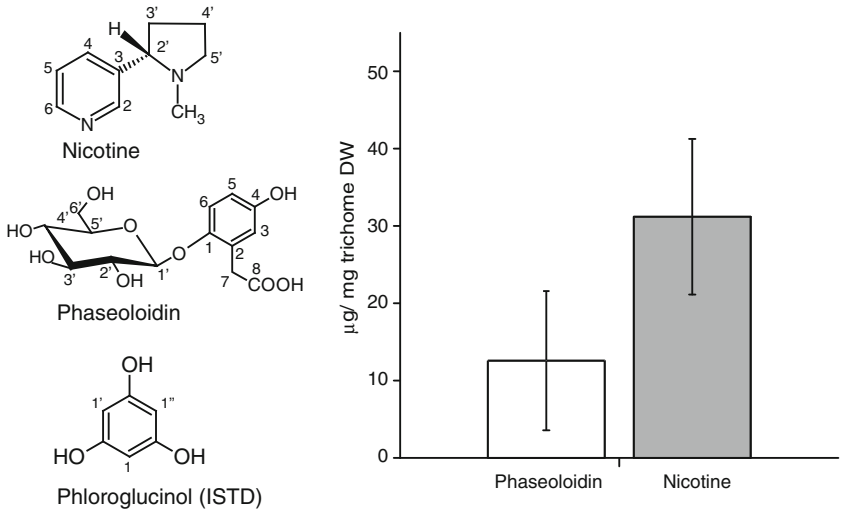


Fig. 1 Quantification of phaseoloidin and nicotine content in *Nicotiana attenuata* wild type trichomes was achieved by ^{1}H NMR. Total amounts of both metabolites were calculated relatively to phloroglucinol (ISTD) according to Formulae 1–2 (below). The integrals of the following proton signals were summed: phaseoloidin: No. of nuclei 3; H-6, H-3, H-5; nicotine: No. of nuclei 4; H-2, H-6, H-4, H-5; phloroglucinol: No. of nuclei 3; H-1, H-1′, H-1″. Error bars indicate standard errors (N=3)

Formula 1: Calculation of the absolute phaseoloidin content

$$m_{phaseoloidin} = \frac{\text{No. of nuclei}_{ISTD} \times n_{ISTD} \times \text{Area}_{phaseoloidin}}{\text{No. of nuclei}_{phaseoloidin} \times \text{Area}_{ISTD}} \times M_{phaseoloidin}$$

Formula 2: Calculation of the absolute nicotine content

$$m_{nicotine} = \frac{\text{No. of nuclei}_{ISTD} \times n_{ISTD} \times \text{Area}_{nicotine}}{\text{No. of nuclei}_{nicotine} \times \text{Area}_{ISTD}} \times M_{nicotine}$$

Abbreviations: m—mass; M—molar mass; n—amount of substance; Area—Integrated area of the NMR peak; ISTD—internal standard (Phloroglucinol)

further purified by preparative HPLC (LiChrospher RP18, 5 µm, 250×10 mm, respectively, *Merck*, Darmstadt, Germany) with the following solvents A (0.1% trifluoroacetic acid in H_2O) and B (100% MeOH) and a linear gradient from 3% B to 30% B in 15 min. The flow rate was 5 ml min^{-1} and a total of 50.0 mg pure phaseoloidin were isolated.

NMR—Experiments ^{1}H NMR, ^{13}C NMR, APT, ^{1}H,^{1}H-COSY, ROESY, HMBC, and HSQC spectra were recorded on a Bruker AVANCE 500 spectrometer (Bruker, Rheinstetten Germany) operating at a ^{1}H frequency of 500.13 MHz and a ^{13}C frequency of 125.76 MHz at 300 K using a TCI Cryoprobe™ (5 mm). Presaturation was used for suppression of the HDO signal in the 1D ^{1}H NMR spectra. Chemical shifts were referenced to internal tetramethylsilane (TMS) for ^{1}H and ^{13}C resonances.

LC-TOF-MS Analysis Prior to NMR measurements, the samples were separated using an Agilent HPLC 1100 Series system, with a Gemini NX C18 column (150×2 mm) and the following binary gradient: 0 to 6 min, isocratic 70% A (deionized water, 0.1% [v/v] acetonitrile [Baker HPLC grade] and 0.05% formic acid), 30% B (acetonitrile and 0.05% formic acid); 6 to 13 min, isocratic 20% A, 80% B; and 70% A, 30% B; 13 to 18 min, isocratic 70% A, 30% B. The flow rate was 200 μl min^{-1}. Eluted compounds were detected by a Bruker MicroToF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ion spray source operated in negative ion mode. The instrument was operated with parameters described in (Heiling et al., 2010). Electrospray ionization conditions: TOF 2100 V, capillary voltage 4500 V, capillary exit −130 V, dry temperature 200°C, dry gas flow of 8 Lmin^{-1}. Mass calibration was performed using sodium formiate (50 ml isopropanol, 200 ml formic acid, and 1 ml 1 M NaOH in water).

Phaseoloidin ^{1}H NMR (MeOH-d_4), 500.13 MHz, internal standard TMS): δ 7.03 (1H, *d*, *J*=8.8 Hz, H-6), 6.70 (1H, *d*, *J*=3.0 Hz, H-3), 6.58 (1H, *dd*, *J*=8.8, 3.0 Hz, H-5), 4.68 (1H, *d*, *J*=7.5 Hz, H-1′), 3.87 (1H, *dd*, *J*=12.1, 5.4 Hz, H-6′a), 3.68 (1H, *dd*, *J*=12.1, 2.2 Hz, H-6′b), 3.56 (1H, *d*, *J*=14.4 Hz, H-7a), 3.46 (1H, *d*, *J*=14.4 Hz, H-7b), 3.45 (1H, *m*, H-2′), 3.44 (1H, *m*, H-3′), 3.42 (1H, *m*, H-4′), 3.33 (1H, *m*, H-5′); ^{13}C NMR (MeOH-d_4, 125.13 MHz, internal standard TMS): δ 179.4 (C-8), 153.7 (C-4), 150.1 (C-1), 130.0 (C-2), 119.0 (C-6), 118.7(C-3), 114.9 (C-5), 104.8 (C-1′), 78.1 (C-5′), 78.0 (C-2′), 75.1 (C-3′), 70.0 (C-4′), 62.7 (C-6′), 39.7 (C-7); HREIMS: *m/z* 331.101 $[M + H]^+$, 329.089 $[M–H]^-$.

Nicotine ^{1}H NMR (MeOH-d_4, 500.13 MHz, internal standard TMS): δ 8.51 (1H, *dd*, *J*=2.2, 0.9 Hz, H-2), 8.44 (1H, *dd*, *J*=5.0, 1.6 Hz, H-6), 7.86 (1H, *ddd*, *J*=7.9, 2.2, 1.6 Hz, H-4), 7.43 (1H, *ddd*, *J*=7.9, 5.0, 0.9 Hz, H-5), 3.24 (1H, *ddd*, *J*=9.6, 7.9, 2.3 Hz, H-5′a), 3.20 (1H, *dd*, *J*=9.1, 8.0 Hz, H-2′), 2.37 (1H, *ddd*, *J*=9.6, 9.3, 8.6 Hz, H-5′b), 2.26 (1H, *m*, H-3′a), 2.17 (3H, *s*, N-CH_3), 1.97 (1H, *m*, H-4′a), 1.89 (1H, *m*, H-4′b), 1.77 (1H, *m*, H-3′b); ^{13}C NMR (MeOH-d_4, 125.13 MHz, internal standard TMS): 149.82

(C-2), 149.15 (C-6), 140.14 (C-3), 137.41 (C-4), 125.40 (C-5), 70.13 (C-2′), 57.90 (C-5′), 40.58 (N-CH_3), 35.73 (C-3′), 23.28 (C-4′).

Caterpillar Bioassays Larvae of *Manduca sexta* (Lepidoptera) were derived from an in-house colony. Larvae of *Spodoptera littoralis* were from Bayer CropScience (Monheim, Germany). *Spodoptera littoralis* larvae were fed prior to the experiments on an artificial diet consisting of 300 g L^{-1} agar, 250 gL^{-1} bean flour, 4.5 g ascorbic acid, 4.5 g ethyl *p*-hydroxybenzoate, 2.5 g vitamin E (all supplied by Sigma, Milan, Italy) dissolved in 17 ml of seed oil and 2 ml formaldehyde.

Artificial diet for feeding experiments was prepared according to (Waldbauer et al., 1964). While the diet was still liquid and cooling, phaseoloidin, homogentisic acid, and nicotine were dissolved in 5 ml MeOH and added to achieve a final concentration of 0.16 μg mg^{-1} diet. As a control, 5 ml of pure MeOH were added. The diet was sealed with plastic wrap and aluminum foil and kept at 5°C until use. Freshly hatched *M. sexta* larvae were transferred to plastic containers containing pieces of artificial diet. Ten caterpillars were placed in one box. Larval mass was measured every 3 day, and the diet was refreshed on these days as well.

Spodoptera littoralis were fed for 5 day on bean flour-containing diet and then transferred to artificial diet amended with either phaseoloidin or nicotine. Because *Spodoptera* larvae are cannibalistic, larvae were kept in single plastic cups with small cubes of diet and wet tissue paper to maintain humid conditions. Caterpillars were weighed and the diet was changed every 3 day.

Results

Trichome Profiling Extracts of the two types of glandular trichomes (long stalked type C and short stalked type D) present on the leaves on *N. attenuata* (Supplemental Figs. S1A, B) were analyzed by NMR. We chose to analyze both trichomes types because of their difference in cell number and distributions on the leaf surface. An insertional mutant with enlarged trichomes allowed for the efficient harvests of type D trichome heads and the middle and tip part of type C trichomes (Supplemental Fig. S1). NMR analysis of the trichome extracts from the transformed plants showed the presence of nicotine, glucose (α, β), fatty acids, and phaseoloidin. The fatty acid and sugar signals most likely result from the well-described *O*-acyl sugars present within trichomes and their exudates (Arrendale et al., 1990). A small amount of phaseoloidin, a β-glucoside of homogentisic acid, was isolated by HPLC from *N. attenuata* trichomes and the structure was unambiguously identified by 1D- and 2D NMR spectroscopy and LC-TOF-MS. ^{1}H and ^{13}C-NMR data are identical with those reported in the literature (Barua et al., 1988). We could not detect phaseoloidin in MeOH/H_2O extracts of entire leaves in our studies using NMR in positive nor negative mode LC-MS-TOF analysis. ^{1}H NMR of trichomes revealed a variable distribution of the metabolites within the trichomes. The nicotine: phaseoloidin ratio across all trichome types and segments within wild type plants of *N. attenuata* is approximately 2: 1 (Fig. 1). There was a clear difference in the amount of nicotine between the different type of trichomes as well as the different trichome tissues dissected (Fig. 2b). Nicotine was distributed in C Tip: C Middle: D Head in an approximate 4: 1: 1 ratio, as calculated by assuming the same water content for all cells and using the water signal for normalization. While the exact ratio varied across different measurements, the overall trend of higher nicotine contents in the tip and middle of type C trichomes remained constant across all measurements. In contrast to the pattern observed for nicotine, the concentration of phaseoloidin was equivalent in the middle and the tip of type C trichomes, where it was three times less than in the head of type D trichomes. The analysis of trichome extracts by LC-TOF-MS showed a slightly different pattern because the amount of phaseoloidin is, in contrast to the NMR- results, higher in the middle part of type C trichomes. Nevertheless the overall finding of lower amounts in type D trichomes was confirmed (Fig. 3a). The ratio of nicotine: phaseoloidin in typical samples was 3: 1 in the trichome heads of type D, 1: 1 in the middle and in 4: 1 tip segments (including heads) of type C trichomes. Additional experiments were conducted on wild type *N. attenuata* plants that revealed no difference in phaseoloidin content compared to the insertional mutant, irGAL83 (Supplemental Fig. S2). Interestingly there was as well a difference in the *O*-acyl sugar distribution between both types of trichomes. The aliphatic part of the NMR- spectra showed much more intense signals corresponding to sugars and fatty acids for type D heads than for type C middle and tip (Supplemental Fig. S3).

Isolation and Identification of Phaseoloidin Due to the fact that the amount of phaseoloidin extractable from the trichomes of *N. attenuata* was insufficient for a bioassay, preparative isolation was performed from the seeds of *Entada phaseolides*. The defatted ground seeds were extracted with aqueous ethanol. The alcoholic extract was partitioned against water, *n*-hexane, ethyl acetate, and *n*-butanol. The ethyl acetate was subjected to two successive column chromatography separations using silica gel followed by semi-preparative HPLC to yield pure phaseoloidin. The structure was identified by 1D- and 2D NMR spectroscopy and HR-TOFMS at *m/z* 329.087 [M-H],

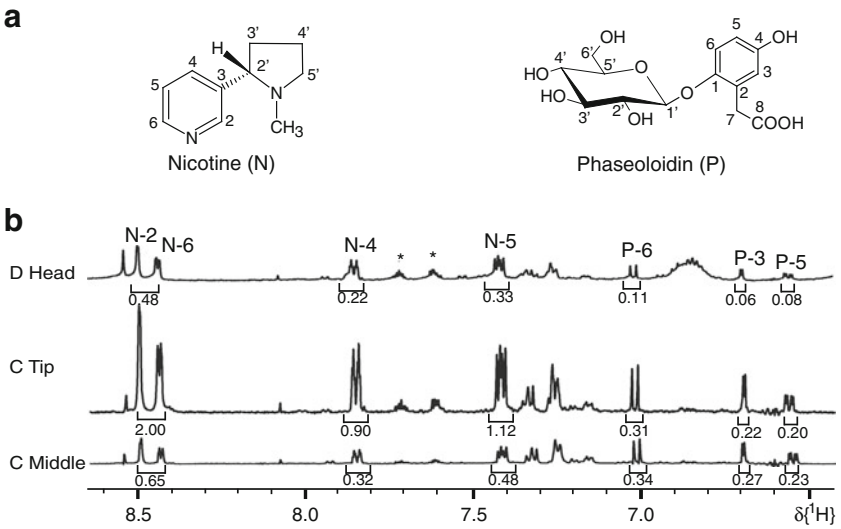


Fig. 2 **a** Structures of nicotine and phaseoloidin found in the two different trichome types of *Nicotiana attenuata*. The identity of phaseoloidin was verified by NMR and LC-ToFMS [M-H 329.0878] with a standard isolated from the seeds of *Entada phaseoloides*. **b** ^{1}H NMR spectra (500 MHz, MeOH-d_4) of head (type D), tip, and middle sections of extracts of tall trichomes (type C). Assuming the same water content of all trichome cells, the integral of the water signal (not shown) was used to determine the relative levels of phaseoloidin and nicotine in the middle and the tip (including the head) sections of type C trichomes and the head of type D trichomes. Typically the ratio of nicotine in sections C Tip: C Middle: D Head was approximately 4: 1: 1 The phaseoloidin concentrations are equivalent in the middle of type C and the head of type D trichomes, which, in turn, are three times less than in the tip of type C. Additional signals in the range of 7.4–7.1 marked with an *asterisk* (*) are from a contamination (phthalate)

corresponding to the molecular formula of $C_{14}H_{18}O_9$ (Fig. 3b, c). The ^{1}H and ^{13}C-NMR data are identical with those reported in the literature (Barua et al., 1988). The MS fragmentation pattern verified the previous results (Fig. 3b, c).

Phaseoloidin Decreases Caterpillar Growth We tested the effects of phaseoloidin and nicotine against the larvae of a generalist herbivore, *Spodoptera littoralis.* Because this insect is known be strongly affected by nicotine, we conducted an assay with this neurotoxin as a comparison. As trichomes are one of the first feeding choices of freshly hatched larvae (Weinhold and Baldwin, 2011), it is likely that trichome-derived compounds are ingested by the caterpillars. Both substances were added to an artificial diet at a final concentration of 0.16 μg mg^{-1}, a concentration approximately 100 times less than that found within the trichomes (Fig. 1). As expected, caterpillars showed decreases in growth of about 45% when fed a nicotine-containing diet. Interestingly they showed a similar decrease in growth when fed on phaseoloidin-containing diets. This response was marginally more pronounced (55% decrease) than the response to nicotine, although the differences were not statistically significant (Fig. 4).

The most damaging insect herbivore on *N. attenuata* frequently is the tobacco hornworm *M. sexta*, and we conducted the same bioassay with this insect species. In addition, we tested the aglycone of phaseoloidin, homogentisic acid. To examine possible synergistic effects of both trichome-derived compounds, we tested the combinations of nicotine and phaseoloidin and nicotine in combination with homogentisic acid. Similar to the response of the generalist, *M. sexta* larvae grew significantly less on diets with either phaseoloidin or nicotine (50 and 37% decreases in growth). Furthermore we observed the same decrease in caterpillar mass for the aglycone as well as for both combinations of phaseoloidin and the aglycone with nicotine (Fig. 4).

Discussion

Trichomes and especially trichome-derived compounds are defensive traits that protect plants from attacking herbivores or pathogens, and recent reports have shown that trichome cells are highly specialized in their ability to synthesize metabolites (Dai et al., 2010). Here, we showed that metabolites contributing to defense occur in *N. attenuata* trichomes in fairly high concentrations (1% of dry mass) and report the presence of a homogentisic acid glucoside, phaseoloidin, in two different trichome types of a Solanaceous plant. Phaseoloidin was found previously within the seeds of *Entada phaseoloides*, a putative function of this substance remained elusive even though Barua et al. (1988) showed a toxicity against fish at fairly high concentrations (125 mg ml^{-1}). Since phaseoloidin was not detectable in complete leaf extracts, we infer that this metabolite occurs primarily in the trichomes, at relatively high concentrations

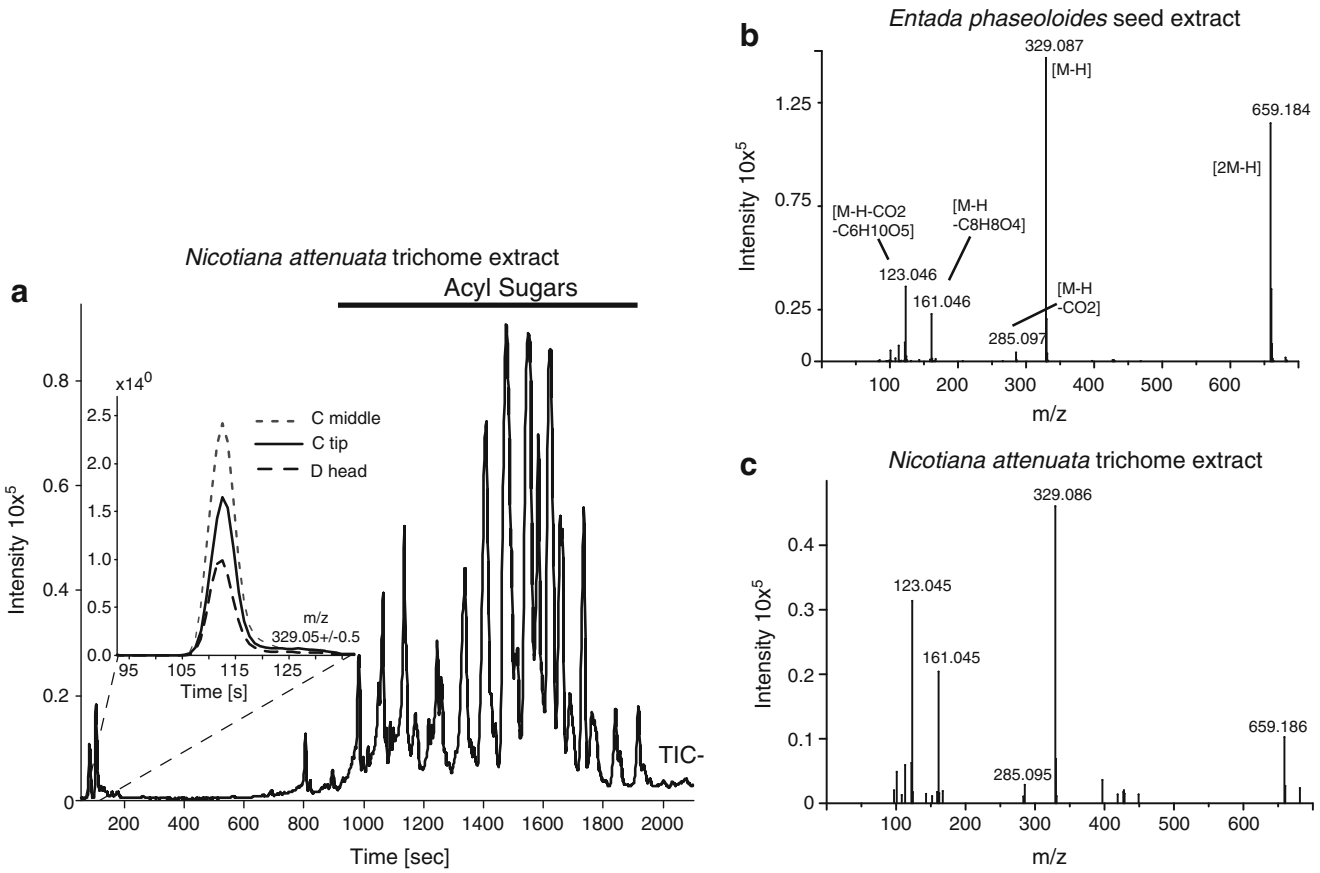


Fig. 3 **a** TIC- (negative total ion current) of a methanolic extract of excised type C and D trichomes from *Nicotiana attenuata*. The TIC is dominated by *O*-acyl sugars (MS spectra not shown) but the phaseoloidin and the differences in the amount between the different tissues can be clearly observed in the ion trace of 329.087 (Inset). **b** Negative ionization mass spectra of phaseoloidin from *Entada phaseoloides* seeds. [M-H] peak is clearly visible (*m/z* 329.087) as well as the loss of CO_2 from the carboxy group of the aglycone (*m/z* 285.097). The fragments at *m/z* 123.045 and *m/z* 161.045 originate from the cleavage of the glycosidic bond. **c** Negative ionization mass spectra of *N. attenuata* trichome extracts confirm the presence of phaseoloidin in this tissue

of approximately 10 μg mg^{-1} dry mass (Fig. 1). These findings suggest that phaseoloidin and nicotine occur in both trichome types present on *N. attenuata's* leaf surface.

The variation in the amounts of metabolites between both types of trichomes as well as among the different trichome parts may be due to variable cell numbers and size but could as well be a consequence of a different biosynthetic site for both metabolites. It is known that nicotine is produced within the roots (Shoji et al., 2000) of *Nicotiana* species upon attack (Baldwin, 1989) and then transported via xylem to leaves, flowers, and trichomes. This transport is mediated by a multidrug and toxic compound extrusion (MATE) transporter as shown for *Nicotiana tabacum* (Morita et al., 2009). In contrast, the biosynthetic site of the phaseoloidin remains unknown. Phaseoloidin is a glucoside of homogentisic acid, which is derived from phenylalanine or tyrosine via 4-hydroxyphenylpyruvate dioxygenase. This enzyme has been described for many plant systems, and so far has not been shown to be present in trichomes (Moran, 2005).

Since phaseoloidin could not be detected in leaf tissue, it most likely is synthesized in the trichomes. Thus, the presence of nicotine and phaseoloidin is an interesting case in which two metabolites differing in their origin are found in trichomes. This tissue type is considered to be highly specific, and there are reports of secondary metabolites specifically synthesized in the trichomes. Examples are the production of artemisinin within trichomes of *Artemisia annua* (Duke et al., 2000), cannabinoids in *Cannabis sativa* (Sirikantaramas et al., 2005), and as well the specific terpenoids in trichomes of *Solanum lycopersicon* (van Schie et al., 2007). Since recent reports show that MATE transporter might be involved in the transport of nicotine, the silencing of the transport of nicotine into the trichomes may show how phaseoloidin acts in the absence of nicotine.

Many trichome-derived compounds have been reported to play a defensive role against herbivores. Our artificial diet bioassays showed that this also holds true for phaseoloidin. This compound decreased caterpillar performance as much as the known defensive compound,

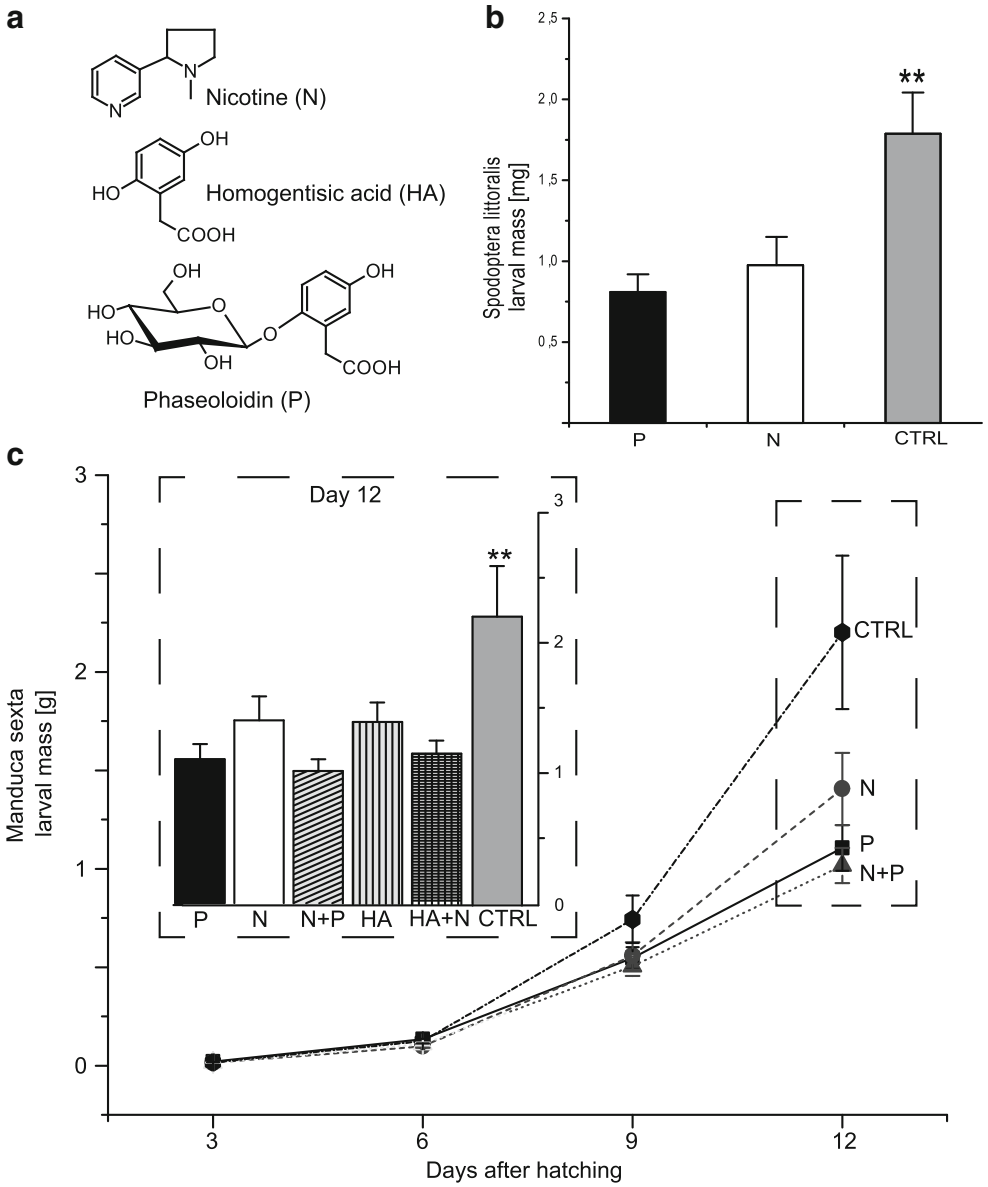


Fig. 4 **a** Structure of compounds used in the bioassay were nicotine (N), phaseoloidin (P) and its aglycone, homogentisic acid (HA). Compounds were dissolved in 5 ml methanol and mixed with freshly prepared artificial diet. As a control (CTRL) diet mixed with 5 ml pure methanol was used. Caterpillars of two different lepidopteran species were fed the amended artificial diet (Waldbauer et al., 1964) to which either nicotine or phaseoloidin in concentrations of 0.16 μg mg^{-1} were added. Larval mass was measured after 8 day for *Spodoptera littoralis* **b** and after 12 day for *Manduca sexta*. Insert shows the larval mass of *M. sexta* after 12 day feeding on artificial diet **c**. Error bars indicate standard errors. *Asterisks* indicate significance levels according to a *Fischer PLSD* * <0.05 ** <0.01 *** <0.001

nicotine. We are aware that artificial diet assays do not reflect the true nature of a plant-herbivore interaction and that silencing the biosynthesis of the target compound would be a more elegant way to examine our hypothesis. Nevertheless, this experimental set-up allows us to study the influence of a single, highly abundant, metabolite without interference from other plant metabolites or proteins. The outcome of these assays shows clearly that phaseoloidin is active against Lepidopteran insects, and ingestion of this homogentisic acid glucoside significantly compromises the performance of generalist and specialist herbivores. Since the effect of phaseoloidin feeding was as pronounced as for the well-known defense metabolite, nicotine, we propose that phaseoloidin functions as a direct defense. From this finding, we hypothesize that diverse functionality of the two trichome types could additionally be responsible for the different ratios of nicotine and phaseoloidin. As has been shown previously, trichomes contribute to plant defense by exuding acyl sugars. These exudates are known to play a major part in plant defense against herbivores, e.g., tagging caterpillars with a distinct body odor that attracts predatory ants (Weinhold and Baldwin, 2011). Hence, it is not surprising that the phaseoloidin concentration is lowest in type D trichomes where the most acyl sugars are exuded (Supplemental Fig. S3). As a consequence, caterpillars may be not deterred by either phaseoloidin or nicotine and can thus feed more intensively on acyl sugars, which leads finally to their predation. In the case of the type C trichomes, the opposite situation occurs; the tips exude less acyl sugars than type D trichomes, but have 3–4 fold higher nicotine and phaseoloidin concentrations. We propose that in type C trichomes,

these metabolites act synergistically as deterrents and toxins for attacking herbivores. In addition to their role in plant defenses, trichomes are known to play a role in the sequestration of phytotoxins (Sirikantaramas et al., 2008). Therefore, we cannot exclude the hypothesis that phaseoloidin might be a detoxification catabolite.

Glandular hairs or trichomes frequently function as physical obstacles and entrap insects by the exudation of glue-like substances, such as *O*-acyl sugars. We propose that caterpillars experience the deterrent effects of phaseoloidin when they navigate around these physical barriers on the leaf surface and become coated with trichome exudates or directly ingest trichome contents. Since phaseoloidin and its aglycone, homogentisic acid, had similar effects, it is possible that the aglycone, or a metabolic product, is itself the active substance. Further experiments with a bioassay-guided fractionation approach may highlight the important defensive function of this new metabolite in the Solanaceae.

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larvae of an Apiaceae-feeding swallowtail species, *Papilio polyxenes* (*ajax*). Despite this early finding, the chemical basis of feeding responses to host chemicals by papilionid larvae remains largely unknown.

Many swallowtail species in the genus *Papilio* utilize host plants in the family Rutaceae. Larvae of *P. xuthus* L. feed on various *Citrus* species and plants in other rutaceous genera such as *Poncirus*, *Zanthoxylum*, *Fagara*, and *Phellodendron* (Honda and Hayashi, 1995). While the oviposition stimulants of *P. xuthus* are composed of a complex mixture of flavonoids, alkaloids, and polar compounds acting synergistically (Nishida et al., 1987; Ohsugi et al., 1991), the specific composition of the larval feeding stimulants remains unknown. The relationship between oviposition preference and offspring performance is a central issue in the evolution of host adaptation in swallowtail butterflies (Wiklund, 1975; Berenbaum and Feeny, 2008). In this connection, a comparison of the chemosensory bases of responses to host-plant cues for adult oviposition and larval feeding in the same swallowtail species could provide insight into divergent selective pressures that act on the adult and larval stages. This report describes the chemical elucidation of the complex larval feeding stimulant system for *P. xuthus* contained in the leaves of one of its favorite hosts, *C. unshiu*.

Methods and Materials

Insects Eggs of *P. xuthus* were obtained from gravid females captured in the city of Kyoto, Japan. Larvae were raised on leaves of *C. unshiu* and other *Citrus* plants at 24± 2°C under a 16:8 hr, L:D photoperiod.

Bioassay Feeding stimulants were bioassayed after application to strips of two-ply toilet paper (Nepia premium soft roll double, Oji Nepia Co., Ltd., Tokyo, Japan, 20× 10 mm). Fifth instars of *P. xuthus* readily bit into and chewed the edges of the strips when these were treated with a crude extract of *C. unshiu*, sometimes biting off much of the edge material. They did not, however, consume significant areas of the strips, comparable to those eaten when feeding on a host-plant leaf. Our bioassay, therefore, was designed to isolate and identify attractants and stimulants responsible for the initiation of feeding. Other components, such as the "swallowing factors" identified by Hamamura and Naito (1961); Hamamura et al. 1962 as stimulants for larval feeding by silkworm larvae, *Bombyx mori*, on mulberry diets, may be required for continued food ingestion by *P. xuthus* larvae.

Each test extract, fraction or mixture of test compounds was applied at a given concentration to the test strip with a micro syringe. A similar volume of control solution (typically a solvent blank or a sugar blend) was added to a separate, control, strip. Doses were expressed as gram-leaf equivalents (g.l.e.) per test strip (200 mm^2). A last-instar larva of *P. xuthus* (12~48 hr after molting) was introduced into a Petri dish (20×90 mm i.d.) containing the test strip and a corresponding control strip (solvent blank). Dishes were kept for 24 hr (24±2°C, 16:8 hr, D:L photoperiod), after which feeding damage was measured. Since the larvae initiated feeding at the margins of the strips, the activity of a particular test sample could be estimated as the area of edge bitten off. Edge was considered to be a 1 mm wide band around the strip, 56 mm in length. There were thus 56 edge sections, each 1 mm^2 in area per ply, and a total of 112 sections per two-ply strip. Feeding was measured as number of sections losing more than half their area (0.5 mm^2) to insect biting and chewing activity. Differences in responses (counts) between the test and control strips were compared by a Wilcoxon rank sum test.

Instruments for Chemical Analysis Preparative HPLC was carried out at 40°C and monitored either with an SPD-20A UV/VIS detector (Shimadzu Corporation, Kyoto, Japan) or an RI-96 refractive index detector (Labo System, Tokyo, Japan). Negative and positive electrospray ionization-mass spectral (ESI-MS) measurements were carried out with an LCMS-2010EV instrument combined with an HPLC system (Shimadzu Corporation), using a reversed-phase column (Mightysil RP-18 GP 75–3.0, 3 μm; Kanto Chemical Co. Inc., Tokyo, Japan) eluted (0.2 ml/min) with a solvent gradient of 20–95% acetonitrile in water over 15 min at a column oven temperature of 40°C. High resolution-electron ionization (HR-EI)-MS data were obtained on a JEOL JMS700 mass spectrometer (Tokyo, Japan). Proton and carbon-13 nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra were measured with a Bruker Avance 400 FT-NMR (Yokohama, Japan) and JEOL JNM-ECP 500 (Tokyo, Japan) using TMS as an internal standard (*J*-values given in Hz).

Extraction and Fractionation Fresh leaves of *C. unshiu* (Miyagawa-wase) were collected in Wakayama Prefecture in August 2005 and extracted with ethanol. The ethanolic extract (2.5 kg.l.e.) was dissolved in water and washed sequentially with ether and ethyl acetate (Fig. 1). The water layer (30.0 g, 500 g.l.e.) was chromatographed on a reverse-phase column (Cosmosil 140C18-OPN, 140 μm, Nacalai tesque, Inc., Kyoto, Japan), eluted with water (Fr. 1A, yield 17.9 g), 10% methanol (Fr. 1B, 771 mg), 20% methanol (Fr. 1C, 1.02 g), 40% methanol (Fr. 1D, 1.84 g), 60% methanol (Fr. 1E, 563 mg), 80% methanol (Fr. 1F, 87 mg), and methanol (Fr. 1G, 363 mg). Fr. 1E was rechromatographed on a reverse-phase column (Cosmosil

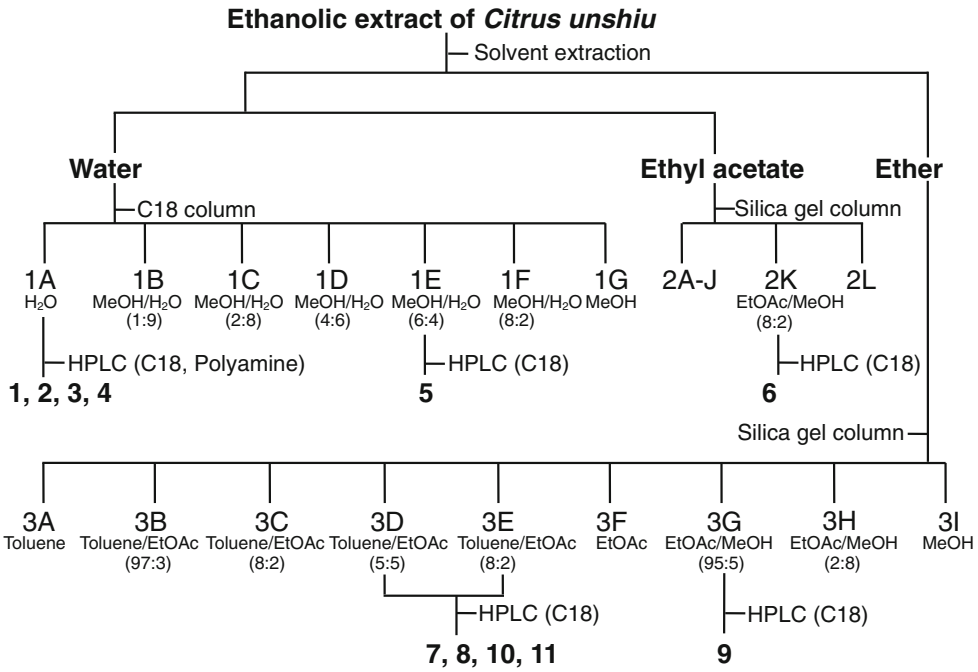


Fig. 1 Procedure for separating feeding stimulants (**1**–**11**) for *Papilio xuthus* larvae from an ethanolic extract of *Citrus unshiu* leaves

140C18-OPN, 140 μm), to separate it into 13 fractions, eluted with a solvent gradient of 40–60% methanol in water.

The ethyl acetate layer (1.76 g, 1 kg.l.e.) was chromatographed on a normal-phase silica gel column (Wakogel C-200, 75–150 μm, Wako Pure Chemical Industries, Ltd., Osaka, Japan), eluted successively with toluene (Fr. 2A, 24 mg), 2% ethyl acetate/toluene (Fr. 2B, 21 mg), 5% ethyl acetate/toluene (Fr. 2C, 17 mg), 15% ethyl acetate/toluene (Fr. 2D, 37 mg; Fr. 2E, 45 mg), 40% ethyl acetate/toluene (Fr. 2F, 96 mg; Fr. 2G, 42 mg), 75% ethyl acetate/toluene (Fr. 2H, 115 mg), ethyl acetate (Fr. 2I, 132 mg; Fr. 2J, 98 mg), 20% methanol/ethyl acetate (Fr. 2K, 209 mg), and methanol (Fr. 2L, 511 mg).

A portion of the ether layer (9.1 g, 500 g.l.e.) was chromatographed on a silica gel column (Wakogel C-200, 75–150 μm), eluted with toluene (Fr. 3A, 4.05 g), 3% ethyl acetate/toluene (Fr. 3B 143 mg), 20% ethyl acetate/toluene (Fr. 3C, 1.30 g), 50% ethyl acetate/toluene (Fr. 3D, 696 mg), 80% ethyl acetate/toluene (Fr. 3E, 269 mg), ethyl acetate (Fr. 3F, 129 mg), 5% methanol/ethyl acetate (Fr. 3G, 135 mg), 20% methanol/ethyl acetate (Fr. 3H, 1.28 g), and methanol (Fr. 3I, 375 mg).

HPLC Separation of Compounds **1**–**11** Fraction 1A was chromatographed on a reverse phase HPLC column (YMC-Pack ODS-AQ, 250×10 mm i.d., YMC Ltd., Kyoto, Japan) eluted with water at a flow rate of 2.0 ml/min, and separated into two fractions: *R*t (retention time)=0–6.5 min; *R*t=6.5–8.0 min. These were chromatographed further on a YMC-Pack Polyamine II column (250×10 mm i.d., YMC Ltd.) with 75% acetonitrile at a flow rate of 2.0 ml/min. Compound **1** was isolated at *R*t=37.0 min (2.8 mg/g.l.e.); **2** at *R*t=30.0 min (1.0 mg/g.l.e.); **3** at *R*t=54.5 min (2.1 mg/g.l.e.); **4** at *R*t=20.5 min (0.79 mg/g.l.e.). Fr. 1E (60% methanol eluate, 26 mg) was chromatographed on a reverse phase HPLC column (YMC-Pack ODS-AM, 300×10 mm i.d., YMC Ltd.) eluted with 70% methanol in water at a flow rate of 2.0 ml/min, and **5** was isolated at *R*t=17.4 min (10 μg/g.l.e.).

Fraction 2 K was chromatographed on a reverse-phase Prep Pak Cartridge 25×100 mm column (Prep Nova-Pak HR C18, 6 μm, 60Å, Waters, Milford, MA, USA) eluted with 60% methanol in water at a flow rate of 3.0 ml/min, and **6** was isolated at *Rt*=38.0 min (3.8 μg/g.l.e.) (Fig. 1).

A series of lipidic components in Fr. 3D and Fr. 3E were recovered from the ether soluble fractions. They were fractionated by a reverse phase HPLC column (Cosmosil MS-II, 250×20 mm i.d., Nacalai tesque Inc.), eluted with 90% methanol in water at a flow rate of 2.0 ml/min. Compounds **7**, **8**, and **11** were isolated at *R*t=63.0 min (96.0 μg/g.l.e.), *R*t=79.0 min (13.2 μg/g.l.e.) and *R*t=105 min (2.8 μg/g.l.e.), respectively. Compound **10** was obtained mainly from Fr. 3D employing a preparative HPLC column (YMC-Pack Pro C18, 250×10 mm i.d., YMC Ltd.) eluted with 90% methanol in water at a flow rate of 2.0 ml/min [isolated at *R*t=32.5 min (11.8 μg/g.l.e.)]. Fraction 3G was chromatographed on a reverse-phase Prep Pak Cartridge 25×100 mm column (Prep Nova-Pak HR C18, 6 μm, 60Å, Waters) using methanol at a flow rate of 3.0 ml/min, and a fraction in a range of *R*t=28–36 min was isolated. This was further chromatographed on a reverse phase HPLC column (YMC-Pack Pro C18, 250×10 mm i.d., YMC Ltd.), eluted with methanol at a flow rate of

2.0 ml/min. Compound **9** was isolated at *R*t=19.1 min (63.4 μg/g.l.e.).

Authentic Samples for Bioassays Compounds **1**, **2**, and **3** (D-glucose, D-fructose, and D-sucrose) were purchased from Wako Pure Chemical Industries, Ltd. Compound **8** (1-linoleoyl-rac-glycerol, approx. 99%) from Sigma Aldrich Japan (Tokyo, Japan). Compounds **10** (glycerol α-monooleate) and **11** (monostearin) were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan), and they were used after purification by HPLC. Other compounds (**4**, **5**, **6**, **7**, and **9**) used for bioassay were obtained from *C. unshiu* leaves (as described above, purity>98%). Standard samples of 1,3-dioleoylglycerol, linolenic acid, and glycerol were purchased from Wako Pure Chemical Industries, Ltd. and trioleoylglycerol (triolein) was purchased from Kanto Chemical Co. Inc.

Results

Larval Feeding Stimulant Activity of Fractions An ethanolic extract of *C. unshiu* was fractionated systematically, and the feeding-stimulant activity of fractions monitored with the paper-strip bioassay, as shown in Fig. 1. The feeding-stimulant activities of the ethanolic extract, ether layer, ethyl acetate layer, and individual chromatographic fractions are shown in Fig. 2. The ethanolic extract showed the highest activity, whereas individual fractions were much less active. Only Fr. 1A and Fr. 1E induced significant feeding responses among the column chromatography fractions (Fr. 1A-1G, Fig. 2). A series of sugars (**1**, **2**, and **3**), amino acids, organic acids (e.g., quinic acid), a basic compound (synephrine), glycerol and an amphoteric compound (**4**) were isolated from Fr. 1A. Sugars **1**, **2**, and **3**, found to be essential ingredients for stimulating larval feeding, were identified as D-glucose, D-fructose, and D-sucrose, respectively (Fig. 3), from their diagnostic ^{1}H-NMR, ^{13}C-NMR spectra and by comparison of these with authentic samples. The activity of the sugar blend (**1**+**2**+**3**) was significantly enhanced by **4** (Fig. 4). Compound **4** was identified as stachydrine by comparison of ^{1}H- and ^{13}C-NMR spectral data (Ohsugi et al., 1991) (Fig. 3). The activity of the sugar blend (**1**+**2**+**3**) was also synergized by compound **5**, isolated from Fr. 1E (Fig. 4), which was identified as a cyclic peptide, citrusin I (Fig. 3).

Citrusin I (**5**): A colorless amorphous powder. ESI-MS *m/z* $[M + H]^+$ 704, ^{1}H-NMR (500 MHz, CD_3OD): δ 0.94 (3H, t, *J*=6.5 Hz), 0.96 (3H, d, *J*=7 Hz), 0.98 (3H, d, *J*=7 Hz), 1.08 (3H, d, *J*=6.5 Hz), 1.16 (3H, d, *J*=6.5 Hz), 1.27 (m), 1.53 (d, *J*=7 Hz), 1.55–1.69 (4H, m), 1.87 (1H, m), 3.01 (1H, dd, *J*=14, 6 Hz), 3.24 (1H, dd, *J*=14, 6 Hz), 3.47 (1H, d, *J*=16.5 Hz), 3.89 (1H, d, *J*=7.5 Hz), 3.99 (1H, m), 4.00 (1H, d, *J*=7 Hz), 4.14 (1H, d, *J*=16.5 Hz), 4.36 (1H, d, *J*=3 Hz), 4.49 (1H, m), 4.53 (2H, d, *J*=3 Hz), 4.81 (1H, d, *J*=6 Hz), 7.26 (5H, m); ^{13}C-NMR (125 MHz, CD_3OD): δ 176.1, 174.8, 174.0, 173.6, 173.6, 173.0, 172.0, 138.6, 131.1, 129.9, 128.1, 70.3, 68.0, 61.1, 60.7, 60.7, 56.2, 53.1, 52.5, 44.4, 41.8, 38.0, 37.4, 27.3, 26.4, 23.9, 21.7, 20.6, 20.4, 16.8, 16.0, 11.6. ^{1}H- and ^{13}C-NMR data are consistent with reports from Matsubara et al. (1991).

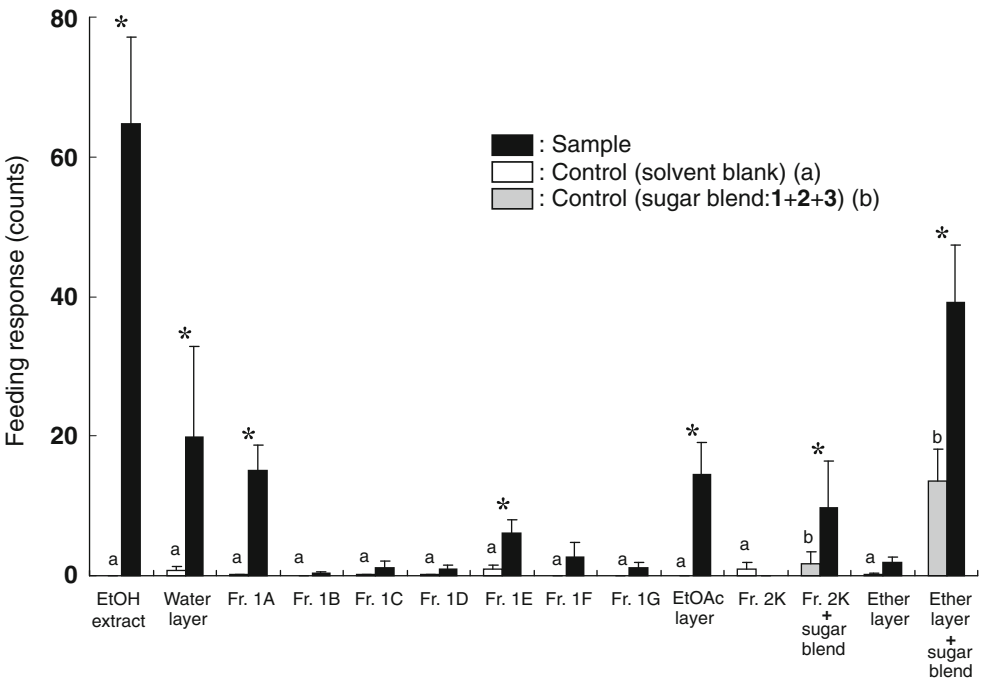


Fig. 2 Feeding stimulant activities of fractions from a *Citrus unshiu* extract in bichoice tests with paper strips a: control (solvent blank), b: control (**1**+**2**+**3**) [Sample dose: 0.05 g leaf equivalent/strip] *: Wilcoxon rank sum test: $P<0.05$, $N=9$ for each paired test, mean ± SE

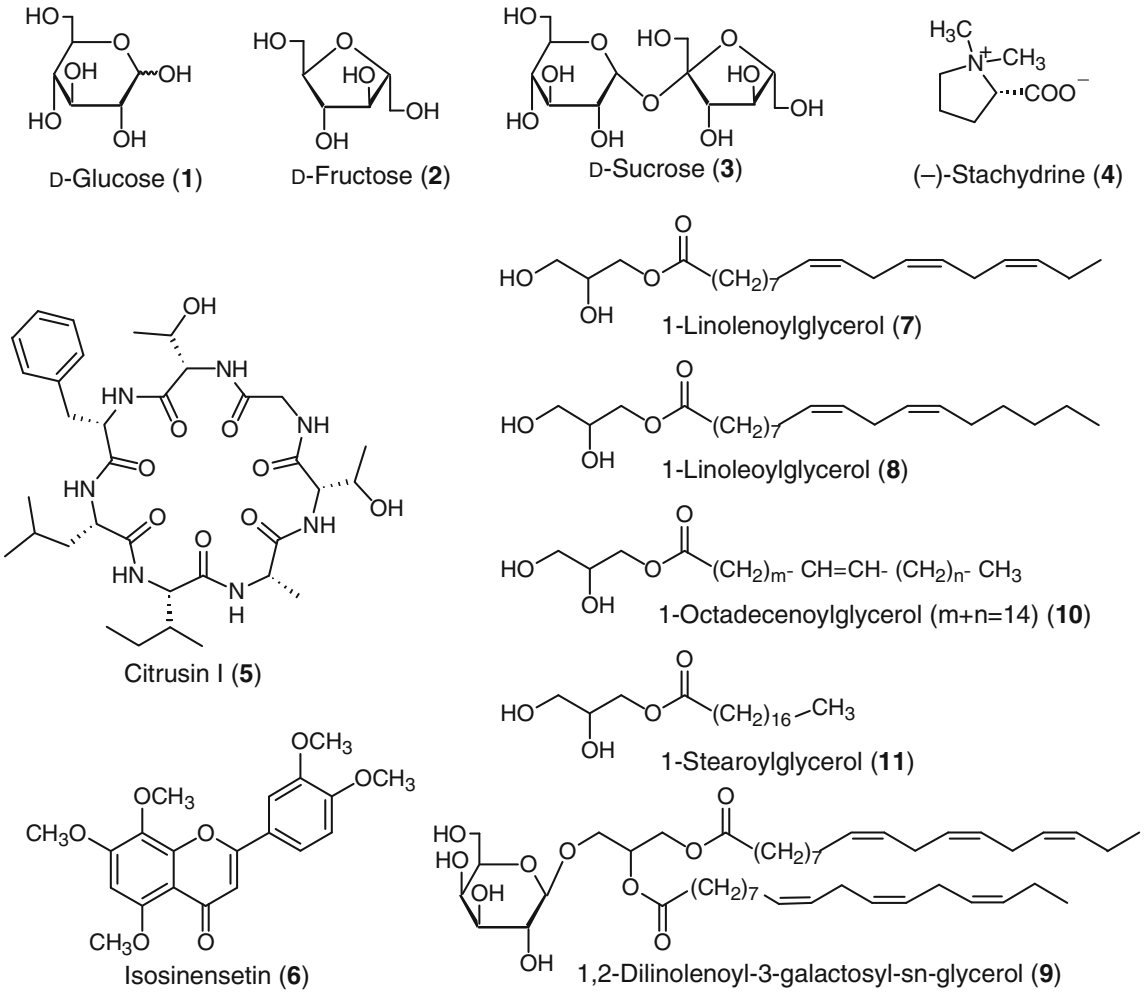


Fig. 3 Structures of larval feeding stimulants (**1**–**11**) of *Papilio xuthus* isolated from leaves of *Citrus unshiu*

Although the ethyl acetate layer showed moderate activity, there were no active fractions among Frs. 2A-2L. When each fraction was tested in combination with the sugar blend, only fraction 2K enhanced activity (Fig. 2). A polymethoxyflavone, isosinensetin (**6**) (Fig. 3), was characterized from fraction 2K and synergized the activity of the sugar blend (**1**+**2**+**3**) significantly (Fig. 4).

Isosinensetin (**6**): A light brown amorphous powder. ESI-MS *m/z* $[M + H]^+$ 373, ^{1}H-NMR (400 MHz, CD_3OD): δ 7.59 (1H, dd, *J*=8.5, 2 Hz), 7.42 (1H, d, *J*=2 Hz), 7.28 (1H, s), 6.99 (1H, d, *J*=8.5 Hz), 4.02 (3H, s), 3.99 (3H, s), 3.98 (3H, s), 3.96 (6H, s). ^{13}C-NMR (100 MHz, CD_3OD): δ 177.8, 160.5, 156.4, 156.2, 151.8, 151.7, 149.1, 130.5, 123.8, 119.4, 111.0, 108.7, 108.3, 106.9, 92.3, 61.3, 56.4, 56.1, 55.9, 55.8. ^{1}H- and ^{13}C-NMR data were consistent with reports by Machida and Osawa (1989).

The activity of a mixture of the six compounds (**1**+**2**+**3**+**4**+**5**+**6**) was appreciably lower than that of the crude ethanolic extract, indicating the presence of one or more additional components in other fractions (Fig. 4). The ether layer, which exhibited marginal activity alone, strongly enhanced activity of the sugar blend (**1**+**2**+**3**) (Fig. 2). After separation on a silica gel column into nine fractions (3A-3I, Fig. 1), the strongest synergistic activity was found in fractions 3D, 3E, and 3 G (Fig. 5). Compounds **7** and **8** were isolated as the two major constituents in fractions 3D and 3E. Compound **9** was characterized from fraction 3G. These compounds, entirely inactive alone, each showed significant activity when mixed with the sugar blend (Fig. 4). Compounds **7**, **8**, and **9** were identified as 1-linolenoylglycerol, 1-linoleoylglycerol, and 1,2-dilinolenoyl-3-galactosyl-sn-glycerol, respectively, by their mass spectra and ^{1}H- and ^{13}C-NMR spectra, as shown below (Fig. 3). In addition, compounds **10** and **11,** relatively minor constituents of fractions 3D and 3E, were identified as a 1-octadecenoylglycerol (tentatively identified as 1-oleoylglycerol without confirmation of the position of unsaturation) and 1-stearoylglycerol by comparison of the spectral data with those of authentic samples of 1-monoolein and 1-monostearine, respectively (Fig. 6).

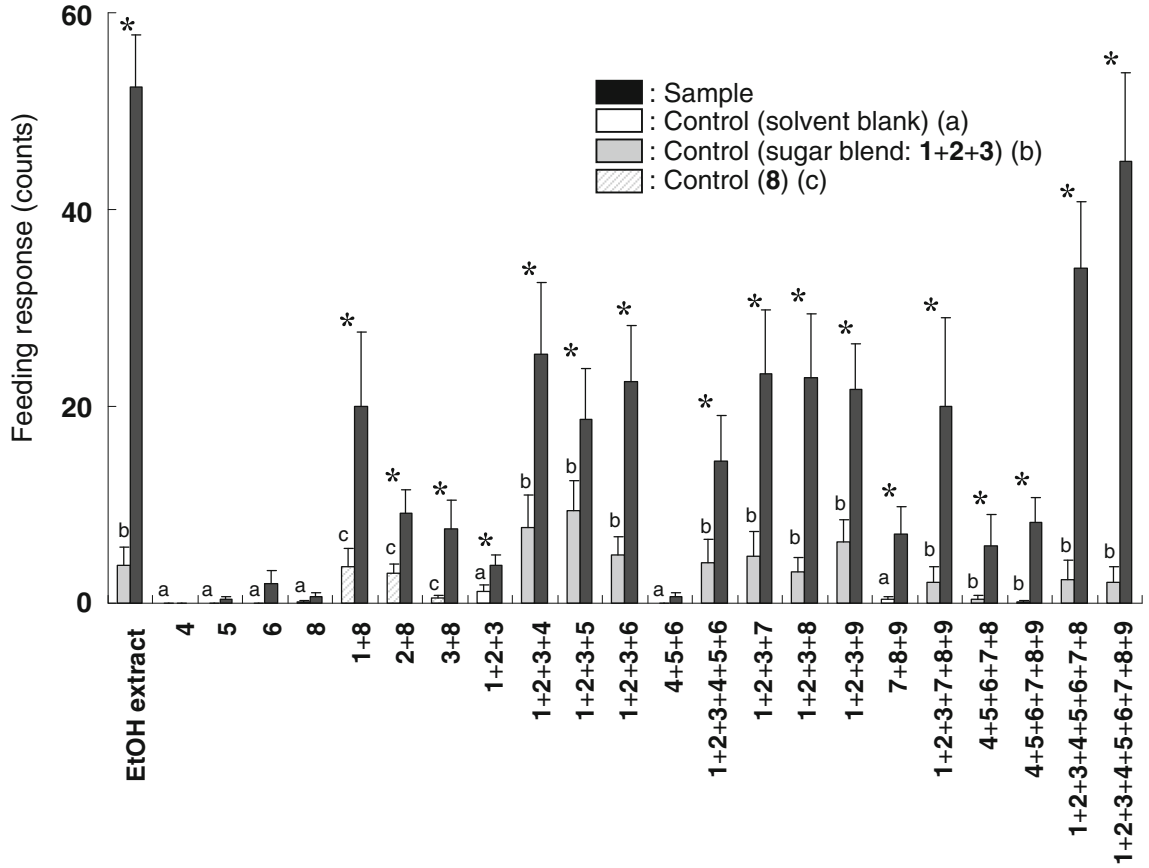


Fig. 4 Feeding stimulant activity of mixtures of compounds **1**–**9** in various combinations in paper strip bioassay. **1**: D-glucose, **2**: D-fructose, **3**: D-sucrose, **4**; (−)-stachydrine, **5**: citrusin I, **6**: isosinensetin, **7**: 1-linolenoylglycerol, **8**: 1-linoleoylglycerol, **9**: 1,2-dilinolenoyl-3-galactosyl-sn-glycerol, a: control (solvent blank), b: control (**1**+**2**+**3**), c: control (**8**) [Sample dose: 0.5 g leaf equivalent/strip] *: Wilcoxon rank sum test: $P<0.05$, $N=9$–27 for each paired test, mean ± SE

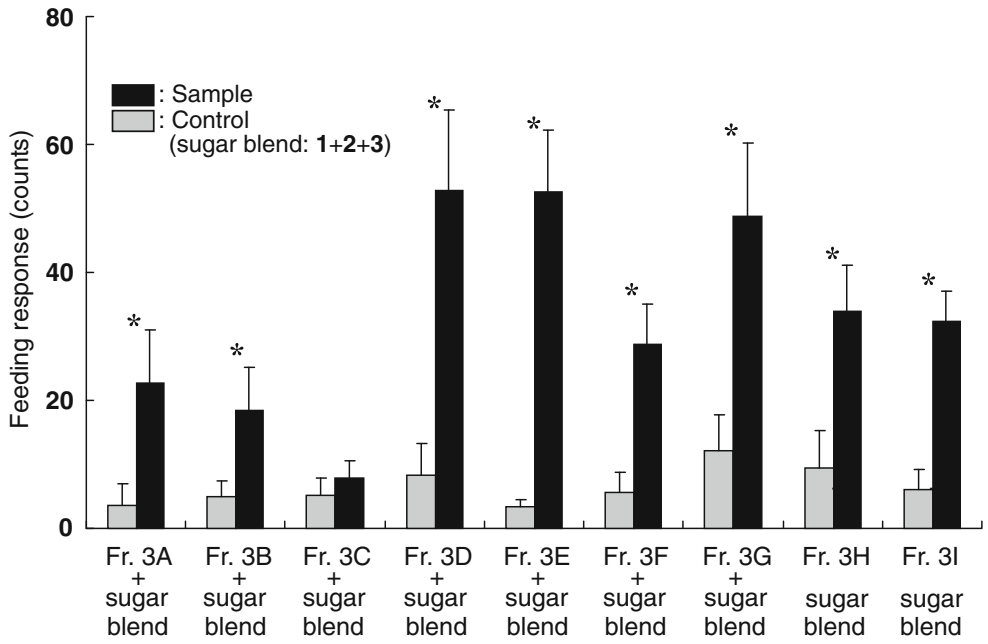


Fig. 5 Feeding stimulant activities of *Citrus unshiu* fractions 3A–3I each containing sugar blend (**1**+**2**+**3**) in bichoice tests with paper strips [Sample dose: 0.5 g leaf equivalent/strip] *: Wilcoxon rank sum test: $P<0.05$, $N=9$ for each paired test, mean ± SE

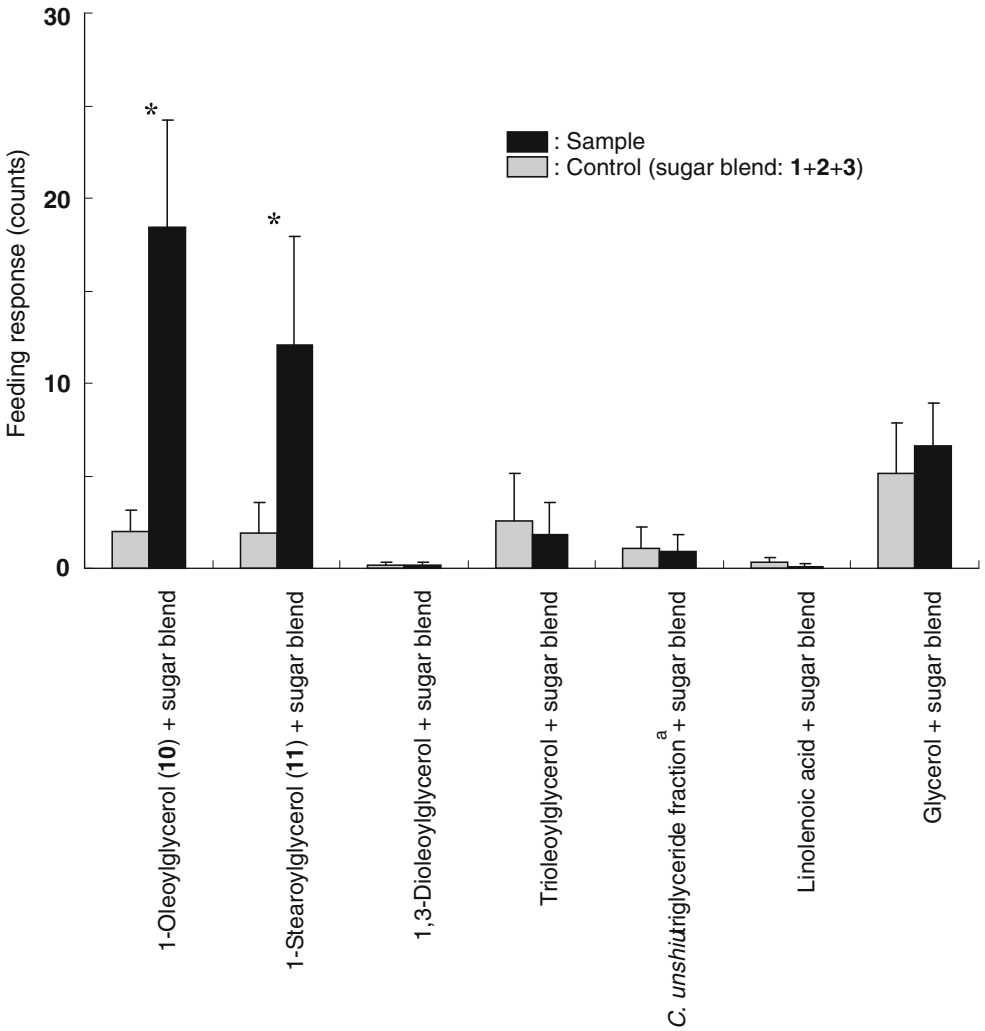


Fig. 6 Feeding stimulant activity of various lipid components present in *C. unshiu* leaves. control; sugars (**1**+**2**+**3**), [Sample dose: 0.5 g leaf equivalent/strip, except 1,3-dioleoylglycerol, trioleoylglycerol, linolenoic acid; each 2.1×10^{-8} mol/strip] *: Wilcoxon rank sum test: $P<0.05$, N=12–15 for each paired test, mean ± SE [a]: obtained from *Citrus unshiu* Frs. 3D + 3E (Fig. 1)

1-Linolenoylglycerol (**7**): A colorless viscous oil. HR-EI-MS *m/z* [M^+] 352.2615 (calcd. for 354.2615). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.34 (6H, m), 4.15 (1H, dd, 12, J= 6.5 Hz), 4.12 (1H, dd, J=12, 6.5 Hz), 3.90 (1H, m), 3.66 (1H, dd, J=11.5, 3.5 Hz), 3.57 (1H, dd, J=11.5, 6 Hz), 2.78 (4H, m), 2.32 (2H, t, J=8 Hz), 2.04 (4H, m), 1.60 (2H, m), 1.20–1.35 (8H, m), 0.95 (3H, t, J=7.5 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$):δ 174.5, 132.0, 130.3, 128.4, 128.3, 127.8, 127.2, 70.3, 65.2, 63.4, 34.2, 29.6, 29.2, 29.2, 29.2, 27.3, 25.7, 25.6, 24.9, 20.6, 14.4. ^{1}H- and ^{13}C-NMR data are consistent with reports by Fraser et al. (2007).

1-Linoleoylglycerol (**8**): A colorless viscous oil. HR-EI-MS *m/z* [M^+] 354.2768 (calcd. for 354.2771). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.36 (4H, m), 4.20 (1H, dd, J=11.5, 4.5 Hz), 4.16 (1H, dd, J=11.5, 6 Hz), 3.93 (1H, m), 3.70 (1H, dd, J=11, 5 Hz), 3.60 (1H, dd, J=11, 5 Hz), 2.77 (2H, t, J=6.5 Hz), 2.35 (2H, t, J=7.5 Hz), 2.05 (4H, m), 1.64 (2H, m), 1.25–1.40 (14H, m), 0.89 (3H, t, J=6.5 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 174.3, 130.2, 130.0, 128.1, 127.9, 70.3, 65.2, 63.3, 34.1, 31.5, 29.6, 29.3, 29.1, 29.1, 29.1, 27.2, 27.2, 25.6, 24.9, 22.6, 14.1. ^{1}H- and ^{13}C-NMR data are consistent with reports from Kim et al. (2003).

1,2-Dilinolenoyl-3-galactosyl-sn-glycerol (**9**): A colorless viscous oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.36 (12H, m), 4.39 (1H, dd, J=12, 3 Hz), 4.26 (1H, d, J=7.5 Hz), 4.20 (1H, dd, J=12, 6.5 Hz), 4.02 (1H, m), 3.91 (1H, dd, J=11, 5 Hz), 3.85 (2H, m), 3.71 (1H, dd, J=11, 6.5 Hz), 3.66 (1H, d, J=7.5 Hz), 3.60 (1H, m), 3.54 (1H, t, J=5.5 Hz), 2.80 (8H, m), 2.31 (4H, m), 2.06 (8H, m), 1.60 (4H, m), 1.30 (16H, m), 0.97 (6H, t, J=7.5 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 173.9, 173.5, 131.9, 130.2, 128.3, 128.2, 127.8, 127.1, 104.0, 74.6, 73.5, 71.3, 70.2, 69.1, 68.1, 62.9, 62.0, 34.3, 34.1, 29.6, 29.6, 29.2, 29.2, 29.1, 29.1, 27.2, 25.6, 25.5, 24.9, 24.8, 20.5, 14.3. ^{1}H- and ^{13}C-NMR data are consistent with reports by Sachdev-Gupta et al. (1993) and Jassbi (2003).

Larval Feeding Stimulant Activity of Constituents The nine major feeding stimulants (**1**–**9**) (Fig. 3), characterized by the systematic bioassay of fractions from *C. unshiu* leaf extracts, were tested in various combinations to assess their

activity to *P. xuthus* larvae (Fig. 4). No individual component stimulated feeding by itself when assayed at the concentration found in the crude ethanolic extract of *C. unshiu* (data for **1**, **2**, **3**, **7**, and **9** not shown in Fig. 4). A mixture of the three sugars (**1**+**2**+**3**) had little activity alone but was strongly synergized by any of the non-sugar compounds (**4**, **5**, **6**, **7**, **8**, and **9**) (Fig. 4). A mixture of **4**, **5**, and **6** was inactive alone but showed moderate activity when combined with the sugar blend (**1**+**2**+**3**+**4**+**5**+**6**). A blend of the three lipids (**7**+**8**+**9**) showed low activity but this was significantly enhanced by addition of the sugar blend (**1**+**2**+**3**) (Fig. 4). The two sugarless mixtures **4**+**5**+**6**+**7**+**8** and **4**+**5**+**6**+**7**+**8**+**9** were both significantly more active than the sugar blend (Fig. 4). A mixture with sugar **1**+**2**+**3**+**4**+**5**+**6**+**7**+**8** showed much higher activity than that of the sugarless mixture (**4**+**5**+**6**+**7**+**8**), (Welch's *t*-test, $P<0.05$). A mixture of all the components (**1**–**9**) showed the highest activity, comparable to that of the crude ethanolic extract (Fig. 4).

In a separate pairwise bioassay, activity of the whole mixture (**1**+**2**+**3**+**4**+**5**+**6**+**7**+**8**+**9**) was compared again with that of the crude ethanolic extract. The activity of the mixture [biting counts (mean ± SD): 37.8±9.0] did not differ significantly from that of the ethanolic extract [biting counts: 38.8±9.9] (Wilcoxon rank sum test $P>0.05$, $N=10$, dose: 0.5 g.l.e./strip) (not shown in Fig. 4).

To examine the synergistic effect of the secondary substances (**4**+**5**+**6**) on the primary sugars (**1**+**2**+**3**) and lipids (**7**+**8**+**9**), feeding responses to the mixture of primary compounds (**1**+**2**+**3**+**7**+**8**+**9**) in the presence and absence of the secondary substances (**4**+**5**+**6**) were compared in a pairwise test. Biting counts were significantly higher in the presence of the secondary substances [mean ± SE: 29.4±5.2] than in their absence [13.5±4.1] (Wilcoxon rank sum test $P<0.05$, $N=12$, dose: 0.5 g.l.e./strip) (not shown in Fig. 4).

Larval Feeding Stimulant Activity of Lipids In addition to compounds **7**, **8**, and **9**, several other lipid constituents of the ether soluble fraction of *C. unshiu* leaves were tested for feeding-stimulant activity with the pairwise paper-strip bioassay. These included the two additional minor monoacylglycerols, **10** and **11** (together accounting for approximately 12% of total 1-monoacylglycerol content), authentic diacylglycerol, triacylglycerol, a free fatty acid, and glycerol, as shown in Fig. 6. Only the monoacylglycerols (**10** and **11**) exhibited significant activity in the presence of sugars (**1**+**2**+**3**) (Fig. 6).

Discussion

In this study, we characterized 11 larval feeding-stimulant components for *P. xuthus* from its major host, *C. unshiu*. These were three sugars (**1**–**3**), a betaine (**4**), a cyclic peptide (**5**), a polymethoxyflavone (**6**), and five lipids (**7**–**11**) (Fig. 3). No individual component alone elicited a feeding response but typical feeding activity was stimulated in varying degrees by the synergistic actions of some of the individual components on the other components of the mixture (Figs. 4 and 6). Sugars (**1**+**2**+**3**) seem to provide a basic taste stimulus, while the rest of the components (**4**–**11**) effectively "seasoned" the sugar taste to varying extents. It also was noted that a mixture of lipids (**7**+**8**+**9**) alone exhibited significant stimulant activity, further enhanced by a mixture of secondary substances (**4**, **5**, and **6**), even in the absence of sugars (Fig. 4).

(−)-Stachydrine (**4**), an effective synergist on sugars (**1**+**2**+**3**), is an amphoteric compound found in quantities as high as 800 μg/g.l.e. in *C. unshiu* leaves. This compound is known to occur especially in *Citrus*, alfalfa, *Chrysanthemum*, and *Stachys* species, and was characterized earlier as one of the oviposition stimulants of *P. xuthus* from *C. unshiu* (Ohsugi et al., 1991, and refs therein).

Citrusin I (**5**) is a unique cyclic peptide composed of seven amino acids that contain a hydrophobic amino acid sequence, phenylalanine-isoleucine-leucine-alanine. Similar cyclic peptides are known as constituents of various *Citrus* species, although their biological function in plants is unknown (Matsubara et al., 1991; Matsumoto et al., 2001, 2002; Morita et al., 2007). Cyclic oligopeptides are known also from other plant taxa (Annonaceae, Caryophyllaceae, Lamiaceae, Linaceae, Rubiaceae, etc.), but their overall distribution in plants is still poorly known.

Isosinensetin (**6**) (3′,4′,5,7,8-pentamethoxyflavone) is one of polymethoxyflavones widely distributed in *Citrus* plants (Machida and Osawa, 1989; Mizuno et al., 1991; Sugiyama et al., 1993; Hirata et al., 2009). Although *C. unshiu* leaves contained several additional polymethoxyflavones, two positional isomers of **6** isolated during fractionation in the present study, namely tangeritin (4′,5,6,7,8-pentamethoxyflavone) and sinensetine (3′,4′,5,6,7,-pentamethoxyflavone), were inactive when compared at the same dose (5 μg/strip) in the presence of sugars (**1**+**2**+**3**). This indicates fairly narrow specificity in the structural requirement for polymethoxyflavone stimulation in *P. xuthus* larvae (T. Murata and R. Nishida, unpublished).

It was surprising that 1-monoacylglycerols (**7**, **8**, **10**, and **11**) and monogalactosyldiacylglycerol (MGDG, **9**) played a central role as potent synergists. 1-Monoacylglycerols are known to coexist with triglycerides as minor components in plants (Dayrit et al., 2008). We have identified lipids **7** and **8** in leaves of another favorite larval host of *P. xuthus*, *Zanthoxylum piperitum* (Rutaceae), in a non-host *Orixa japonica* (Rutaceae), and in carrot *Daucus carota* (Apiaceae) in substantial quantities (unpublished data), suggesting that 1-monoacylglycerols may be present universally among

larval host plants of several *Papilio* species. As shown in Fig. 6, diacyl and triacylglycerols, and corresponding free carboxylic acids, were entirely inactive in combination with sugars, demonstrating the high specificity of the monoacylglycerol structure. The number of double bonds on the fatty acid moiety did not affect the activity (Figs. 4 and 6). MGDG (**9**) is present ubiquitously in the chloroplast as a major membrane lipid consisting of diacylglycerol with a galactosyl moiety (Roughan and Batt, 1969; Joyard et al., 1998; Ohta et al., 2000). Although the molecular structure of **9** has no direct similarity to that of the monoacylglycerols, the compounds may share surfactant properties (Selmair and Koehler, 2008). MGDG was reported as an oviposition stimulant component for a pipevine swallowtail butterfly, *Battus philenor*, together with aristolochic acids (Sachdev-Gupta et al., 1993). Insects are capable of synthesizing many fatty acids and these may not be required as fundamental dietary ingredients, but polyunsaturated fatty acids are essential to many insects as a dietary source (Nation, 2002). The responses to such compounds, as well as to mono- and disaccharides, as feeding stimulants may provide *P. xuthus* larvae with the ability to assess the quality and/or quantity of essential dietary nutrients in plant tissues.

The strong affinity toward essential nutrients can be observed universally throughout the animal kingdom; sugars and amino acids in particular are known as general feeding stimulants in herbivorous insects, where the larvae develop specific receptors tuned for these primary substances (Bernays and Simpson, 1982; Schoonhoven et al., 2005). However, we could not detect any prominent activity in amino acid fractions by using our paper-strip bioassay method. *Citrus unshiu* leaves contain an extraordinarily high quantity of L-proline (0.64 mg/g.l.e.) along with its *N*,*N*-dimethyl analog, stachydrine (**4**), but L-proline exhibited no synergistic activity on the sugar mixture (**1**+**2**+**3**) [biting counts: control (**1**+**2**+**3**) 0.67±0.47, sample (**1**+**2**+**3**+ L-proline 0.89±0.67]. Interestingly, another Rutaceae-feeding swallowtail, *P. protenor*, uses both stachydrine (**4**) and proline as oviposition stimulants in *C. unshiu* (Honda, 1990).

Coincidence of chemical profiles between larval feeding stimulants and oviposition stimulants has been shown in several butterfly taxa. Examples are glucosinolates as stimulants for cabbage butterflies, *Pieris* spp. (Pieridae, host Brassicaceae), iridoid glycosides for the buckeye, *Junonia coenia* (Nymphalidae, host Scrophulariaceae etc.), and aristolochic acids for a pipevine swallowtail, *Atrophaneura alcinous* (Papilionidae, host Aristolochiaceae) (Verschaffelt, 1910; David and Gardiner, 1962; Renwick and Lopez, 1999; Renwick, 2002; Bowers, 1984; Pereyra and Bowers, 1988; Nishida and Fukami, 1989a, b). These patterns suggest a possible congruency of chemosensory mechanism developed in both the gustatory receptors on the mouthparts of larvae and the tarsal receptors of adult females that perceive specific host chemicals. In the case of *P. xuthus*, ten oviposition stimulants identified from *C. unshiu* include flavonoid glycosides (vicenin-2, hesperidin, narirutin, rutin), a nucleoside (adenosine), protoalkaloids (5-hydroxy-*N*ω-methyl-tryptamine, bufotenine, synephrine), a cyclitol (*chiro*-inositol), and a betaine (stachydrine). Only stachydrine (**4**) has been identified as both a larval feeding and an oviposition stimulant. Unlike polymethoxyflavone **6**, flavonoid glycosides with oviposition stimulant activity did not seem to stimulate larval feeding, since the corresponding fractions (i.e., 1C, and 1D in Fig. 1) were totally inactive in the paper strip assay (Fig. 2). We also have tested other major oviposition stimulant components, such as synephrine and *chiro*-inositol, for feeding-stimulant activity but the results appeared negative when tested in combination with sugars (**1**+**2**+**3**) at the given dose (0.5 g.l.e./strip) (unpublished). These findings suggest that adults and larvae of *P. xuthus* may differ in their chemosensory mechanisms for host recognition, although the chemical bases of neither oviposition stimulants nor larval feeding stimulants in the *P. xuthus*—*C. unshiu* association have been fully elucidated (Nishida, 2005). As hypothesized by Wiklund (1975), the difference in host preferences between the egg-laying female and feeding larvae of the Apiaceae-feeding swallowtail, *P. machaon*, may be determined by separate gene complexes. By contrast, however, a congruent chemosensory mechanism in *P. xuthus* has been suggested by the rejection of allomonal chemicals contained in a non-host rutaceous plant, *O. japonica*, where two specific hydroxybenzoic acid derivatives commonly act as potent deterrents on both female tarsal chemoreceptors and larval maxillary taste receptors (Ono et al., 2004; Nishida, 2005).

Although both oviposition and larval feeding are controlled by multiple chemical factors in *P. xuthus*, all of the ten oviposition stimulants are regarded as secondary metabolites. By contrast, of the 11 larval feeding stimulants identified in the present study, only three (**4**, **5,** and **6**) can be considered as secondary while the other eight (**1**, **2**, **3**, **7**, **8**, **9**, **10,** and **11**) are primary metabolites. This contrast in the patterns of chemosensory cues used by larvae and adults presumably reflects divergent selective pressures acting on the two different life-history stages. Reliance by adult females on unique chemical profiles of secondary compounds is likely to facilitate the location and recognition of individual host plants within diverse plant communities. Responses to ubiquitous primary metabolites presumably would be less useful for the females, except perhaps as supplementary cues for leaf choice and egg placement on the host plant. Once the *P. xuthus* female lays an egg on a *Citrus* plant, the hatched larva usually stays on

the same shrub or tree throughout all the larval stages and typically does not seek another food plant. Unique secondary compounds thus may be less critical for larval feeding than are primary compounds such as sugars and lipids that may facilitate the choice of foliage more favorable for larval growth and survival within the host shrub or tree. This contrast in host-recognition profiles for adults and larvae may differ for herbaceous feeders, such as the pipevine swallowtail, *A. alcinous*, whose larvae must seek new host-plant patches after the original plant is entirely consumed. For both larvae and ovipositing females of this species, aristolochic acids function as a major host-finding cue (Nishida and Fukami, 1989a).

The complexities of host recognition and feeding stimulation in lepidopteran larvae initially were demonstrated for the silkworm moth, *Bombyx mori*. Larval feeding behavior in this species is controlled by three distinct factors: attractants (e.g., citral, linalool, 3-hexenol), biting factors (e.g., isoquercitrin, β-sitosterol), and swallowing factor (cellulose) in addition to co-factors (e.g., sucrose, *myo*-inositol, inorganic phosphate) (Hamamura and Naito, 1961, Hamamura et al., 1962). A recent study revealed an olfactory receptor protein responsible for detection of a key mulberry leaf volatile, jasmone (Tanaka et al., 2009). Although our paper-strip bioassay did not provide any indications of an involvement of olfactory cues in feeding by *P. xuthus* larvae, the importance of specific host-plant volatiles prior to feeding has been demonstrated in the lemon butterfly, *P. demoleus* (Saxena and Prabha, 1975). Dethier (1941) found that larvae of the black swallowtail, *P. polyxenes*, were attracted by certain volatiles in plants of the Apiaceae and Rutaceae and speculated that these volatiles may have facilitated host shifts by some *Papilio* species from the Rutaceae to the Apiaceae. Similarly, a resemblance of underlying phytochemical cues appears to have facilitated host shifts from the Apiaceae to the Asteraceae in species of the *Papilio machaon* group (Murphy and Feeny, 2006). Elucidation of olfactory and gustatory chemical cues and their chemosensory mechanisms of host recognition both at egg-laying and larval feeding will contribute to understanding the evolution of host selection in these closely related oligophagous *Papilio* species.

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them (Chen and Dorn, 2009). Despite these similarities, infestation by the highly invasive pest *G. molesta* has not been reported on Juglandaceae or other plants that contain naphthoquinones, in contrast to the codling moth, which thrives on walnut (Phillips and Barnes, 1975; Piskorski and Dorn, 2011). Juglone contents in walnut fluctuate over the season (Radix et al., 1998; Colaric et al., 2005; Solar et al., 2006; Stampar et al., 2006), and survival rates of the codling moth were high at the lower juglone level and moderate at the higher level reported for this plant species. Concentrations of juglone that were double the maximum juglone content in walnut were lethal to the codling moth larvae (Piskorski and Dorn, 2011), indicating that detoxification is limited even in a herbivorous species adapted to walnut (Barnes, 1991) and its naphthoquinones. We hypothesized that the oriental fruit moth may be unable to detoxify juglone, thus limiting its potential to use walnut as a host. However, by using performance bioassays we showed that *G. molesta* can survive on a diet containing juglone, and chemical analysis of larval frass suggests the ability of this species to reduce this compound in its digestive tract.

Methods and Materials

Insects Larvae of the oriental fruit moth were collected from a peach orchard in Trentino, Italy, in 2007 and reared in the laboratory under controlled conditions [16 : 8 hL:D cycle at 26/24°C and 60% relative humidity (r.h.)]. Upon emergence, the adults were transferred to a paper cylinder with moist cotton (Hern and Dorn, 1999). Neonate larvae were transferred to Petri dishes and allowed to feed on an artificial diet as specified below. Corrugated cardboard strips were offered for pupation. Unfed first-instars (24± 12 h-old) were used for bioassays.

Artificial Diet The diet offered to larvae was a soya flour-based artificial diet devoid of any fruit material, used in mass rearing of the oriental fruit moth in our laboratory (Torriani et al., 2010). Juglone was added at the end of diet preparation; the diet was homogenized and distributed into plastic Petri dishes of 55-mm diam to yield a 4-mm thick layer (Piskorski and Dorn, 2011). Juglone (purity≥97%; Sigma-Aldrich, Buchs, Switzerland) was added to the diet at concentrations of 5, 25, and 50 mg/g dry weight (d.w.), with the first two concentrations corresponding to the minimum and maximum of the seasonally fluctuating juglone content in walnut husks (Radix et al., 1998; Stampar et al., 2006; Piskorski and Dorn, 2011). The diet was prepared directly prior to conducting the bioassay. Juglone was stable throughout the diet preparation and bioassay (Piskorski and Dorn, 2011).

Effect of Juglone on G. molesta Performance First-instar larvae of *G. molesta* (N=30–34 per juglone concentration) were placed individually with a fine brush into Petri dishes with artificial diet containing juglone or with juglone-free diet (Piskorski and Dorn, 2011). Insects were allowed to develop under controlled conditions with a 16 : 8 hL:D cycle at 26/24°C and 60% r.h. Corrugated cardboard strips were attached to the inner wall of each Petri dish lid after 7 d of feeding to allow for pupation.

Development was monitored daily from day 7 onwards, i.e., before the larvae were expected to terminate feeding prior to pupation. Larval developmental time was recorded as the number of days from introduction of the larva onto the diet until its entry into the cardboard strip for pupation. Pupal developmental time was recorded as the period between larval entry into the cardboard strip and adult emergence, thus including any non-feeding prepupal stage the larva may have undergone (Notter-Hausmann and Dorn, 2010; Piskorski and Dorn, 2011). Larval survival was assessed as the proportion of individuals that entered the cardboard strip in relation to those that had been introduced as first-instar larvae. Pupal survival was assessed as the proportion of emerged adults in relation to the number of individuals that had entered the strip (Notter-Hausmann and Dorn, 2010; Piskorski and Dorn, 2011).

To track sublethal effects, the weight of adults was recorded upon emergence (Velten et al., 2007; Piskorski and Dorn, 2011); within 24 h of emergence the adults were killed by freezing, dried for 24 h in a 60°C oven, and weighed. To determine the larval weight gain, fresh weights of larvae were recorded on the 9th day of feeding (Piskorski and Dorn, 2011), i.e., when all larvae were still feeding and none had yet entered the non-feeding prepupal stage.

Fate of Juglone To follow the fate of juglone in insects feeding on the diet that contained juglone at 25 mg/g d.w. the following samples were analyzed: (1) frass, which was collected only from larvae (N=8), which successfully entered the pupal stage; (2) larval regurgitant (N=12), collected by gently touching the head with a micropipette and collecting the fluid; (3) larval hemolymph (up to 1 µl from each larva; N=12), collected with a micropipette directly from each insect after cutting its cuticle from head to abdomen on the ventral side; (4) fed late-instars, i.e., larvae containing filled guts (N=12); (5) starved late-instars (N=6), which were placed for 17 h in Petri dishes, devoid of any food, on humid filter paper to empty their gut prior to analysis; (6) pupae (5–7 d-old; N=8); (7) newly emerged adults (N=12) (Piskorski and Dorn, 2011). Samples (2)–(5) were collected after a 9-d-feeding period. Samples (1), (2), and (7) (frass, regurgitant, and adults) were taken from insects subjected to the performance trial, whereas samples (3)–(6) (body tissues) were prepared in a separate trial.

Insects were killed by freezing, freeze-dried, and ground before extraction. Samples of each type were pooled. All samples were extracted twice with a $CHCl_3$:MeOH (2:1) mixture (5 ml, or 0.5 ml for regurgitant and hemolymph, 10 min sonication) (Piskorski and Dorn, 2011), the two extracts were combined, and analyzed with gas chromatography–mass spectrometry (GC-MS).

Chemical Analysis GC-MS analysis was performed with a 6890 GC-5973 MS instrument (Hewlett-Packard, Palo Alto, CA, USA) equipped with a 30 m × 0.25 mm i.d., 0.25 μm, DB-5 ms column (J&W Scientific Inc., Folsom, CA, USA). Helium was used as carrier gas at a constant flow (1.0 ml/min); an aliquot of each sample (1 μl) was injected. The oven temperature was kept at 50°C for 2 min, then increased to 320°C at a rate of 10°C/min, and kept at that temperature for an additional 10 min. Electron ionization (70 eV) mass spectra were recorded with ion source temperature of 230°C, and quadrupole temperature of 150°C. Identification of juglone and 1,4,5-trihydroxynaphthalene was based on chromatographic and mass spectrometric characteristics and confirmed with synthetic standards; 1,4,5-trihydroxynaphthalene was synthesized previously in our laboratory (Piskorski and Dorn, 2011). Linear retention indices (RI) were calculated using *n*-alkanes as standards and argon as an unretained compound (Piskorski et al., 2007). If juglone or related compounds were not detected in a given extract, the extract was concentrated and re-analyzed.

Statistical Analysis Developmental times and larval and adult weights were analyzed across treatments using analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The χ^2-test was used to test for differences in the number of surviving individuals between treatments; survival on the control diet served as baseline value. The statistical analyses were performed with the JMP 8.0 software (SAS Institute Inc., Cary, NC, USA).

Results

Effect of Juglone in the Artificial Diet on G. molesta Performance Juglone concentrations reflecting the minimum of the seasonally fluctuating level in walnuts (5 mg/g d.w.) did not have any direct effect on survival rate of *G. molesta,* whereas concentrations reflecting the maximum level (25 mg/g d.w.) significantly affected larval and pupal survival, reducing the number of individuals that reached adulthood by one third compared to the control (Table 1). Amending the diet with a juglone concentration (50 mg/g d.w.) that reflected twice the maximal level reported from the field completely prevented larval development, and all larvae on that diet died within a few days.

A similar effect was observed for larval developmental time (Table 2). Mean developmental time of larvae feeding on diet supplemented with juglone at a concentration representing the lowest natural levels was not affected. Significantly prolonged development time of larvae fed on diets containing the highest juglone level reported from the field was observed. It lasted nearly twice as long as in the control. Pupal developmental time

Table 1 Percent survival of *Grapholita molesta* reared on the artificial diet containing juglone

Diet	N[a]	Developmental stage	Actual survival rate to the stage[b]	Apparent survival rate during the stage[c]	P[d]
Juglone 5 mg/g	34	Larva	100	94	ns
	32	Pupa	94	97	ns
	31	Adult	91	–	–
Juglone 25 mg/g	32	Larva	100	66	***
	21	Pupa	66	86	*
	18	Adult	56	–	–
Juglone 50 mg/g	32	Larva	100	0	***
	0	Pupa	0	0	–
	0	Adult	0	–	–
Control (= basis for comparison)	30	Larva	100	90	–
	27	Pupa	90	96	–
	26	Adult	87	–	–

[a] The number of individuals entering a given developmental stage

[b] The percentage of individuals that successfully entered the stage in relation to those that had been introduced as first-instar larvae

[c] The percentage of individuals that successfully entered the next developmental stage in relation to those in the respective stage

[d] ns $P \geq 0.05$; * $P < 0.05$; *** $P < 0.001$ (comparison of apparent survival rate with χ^2-test; survival on the control diet served as baseline value)

Table 2 Developmental time of immature stages of *Grapholita molesta* reared on artificial diets containing juglone

	Larva (d)[a]	Pupa (d)[a]
Juglone 5 mg/g	11.7±0.3 (32) a	10.4±0.1 (31) a
Juglone 25 mg/g	19.9±0.9 (21) b	11.1±0.2 (18) a
Juglone 50 mg/g[b]	–	–
Control	10.6±0.2 (27) a	10.1±0.1 (26) a

[a] Mean values±SEM of the number of individuals given in brackets. Different letters after the mean indicate significant differences between treatments in a column at $P<0.05$ (comparison between treatments with ANOVA and Tukey's *post hoc* test)

[b] No development at the larval stage

remained unaffected at both of these juglone concentrations (Table 2).

Sublethal effects of juglone on insect weight were noted. After 9 days, larvae feeding on diets containing juglone had significantly lower weights compared to larvae feeding on the control diet (Table 3). Larval weight was reduced only about 10% by the low juglone diet, but was reduced 95% by levels of juglone similar to the highest levels reported in walnut. Adult weights were reduced approximately 25% for larvae fed diets containing the high level of juglone reported from walnut husks (Table 3).

Fate of Juglone To understand the successful development of *G. molesta* feeding on diet containing juglone, chemical analysis of larval exudates, hemolymph, and insect bodies was performed in search of juglone or its likely metabolites. In larval frass, the reduced form of juglone, 1,4,5-trihydroxynaphthalene, was detected in relatively high concentrations compared to juglone, with a ratio of 1.22 : 1.00 (1,4,5-trihydroxynaphthalene : juglone). In fed larvae, the guts contain food and digesta, and traces of both juglone and its reduced form were detected. In starved larvae, the gut is empty and neither juglone nor its reduced form were present. Furthermore, neither these nor related phenolic or quinone derivatives were detected in larval regurgitant, in larval hemolymph, in pupae, or in adults. Other likely derivatives of juglone were not detected in any of the samples.

Discussion

The oriental fruit moth *G. molesta*, which has not been reported to use plants containing naphthoquinones as hosts, successfully achieved adulthood on diets supplemented with juglone at levels reflecting those found in walnut husks. At juglone concentrations reflecting the minimum and maximum of the seasonally fluctuating levels (Radix et al., 1998; Stampar et al., 2006), survival rates to adulthood were 90% and more than 50%, respectively, of the control survival rate. Survival rates observed in an analogous experiment with the codling moth *C. pomonella*, which readily accepts walnut as a host, were similar at the low juglone concentration, and only approximately 20% higher at the high juglone concentration (Piskorski and Dorn, 2011).

Sublethal effects observed in juglone-fed *G. molesta* consisted of slightly reduced larval weight gain at the low juglone concentration, and prolonged developmental time as well as reduced larval and adult weight at the high juglone concentration as compared to the control. These effects qualitatively resemble those found recently for *C. pomonella* (Piskorski and Dorn, 2011), but they are quantitatively more pronounced in *G molesta*. Development was completely prevented in *G. molesta* at the juglone level reflecting twice the maximum reported from the field, suggesting that the adverse effects were dose dependent. Similarly, none of the *C. pomonella* larvae exposed to this excessively high level of juglone survived (Piskorski and Dorn, 2011), indicating that even in a herbivorous species that includes naphthoquinone-containing plant material in its diet, the capability to cope with these defensive metabolites is limited.

It is surprising that an herbivorous species can tolerate a defensive compound that is not a constituent of its reported hosts. In fact, physiological constraints of plant-feeding insects are thought to be particularly serious limitations to host plant expansion (Praz et al., 2008; Sedivy et al., 2011). Similar to the oriental fruit moth, the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and the gypsy moth *Lymantria dispar* (Lepidoptera: Lymantriidae) have not been observed to feed on plants that contain naphthoquinones. Juglone is lethal for these two lepidopteran larvae at doses as low as 2.0 mg/g (Yu, 1987; Lindroth et al., 1990), which is lower than the lower limit reported for walnut (Radix et al., 1998; Stampar et al., 2006;

Table 3 Larval fresh weight after 9 days of feeding and adult dry weight of *Grapholita molesta* reared on artificial diet containing juglone

	Larva (mg)[a]	Adult (mg)[a]
Juglone 5 mg/g	14.6±2.1 (25) b	3.9±0.2 (31) a
Juglone 25 mg/g	0.9±0.3 (17) c	3.1±0.3 (18) b
Juglone 50 mg/g[b]	–	–
Control	16.1±1.3 (20) a	4.1±0.2 (26) a

[a] Mean values±SEM of the number of individuals given in brackets. Different letters after the mean indicate significant differences between treatments in a column at $P<0.05$ (comparison between treatments with ANOVA and Tukey's *post hoc* test)

[b] No development at the larval stage

Piskorski and Dorn, 2011), and lower than the concentrations tolerated by the oriental fruit moth.

The mechanism of tolerance to juglone by the oriental fruit moth needs further investigation. In other lepidopteran species, redox cycling (Thiboldeaux et al., 1994) or conjugation with glutathione (Thiboldeaux et al., 1998) could not satisfactorily explain the observed effects of 1,4-naphthoquinones. However, enzymatic reduction of the conjugated cyclic 1,4-dione moiety to the corresponding diol offered a good explanation for different susceptibilities of various lepidopteran species. Quinone reductase, which catalyses reduction of juglone, is active in the intestinal system of some lepidopterans (Yu, 1987; Lindroth, 1989). Low levels of quinone reductase activity (maximum 50 nmol/min/mg protein) were found in the fall armyworm and the gypsy moth, which perform poorly on juglone-containing diet (Yu, 1987; Lindroth et al., 1990). In contrast, a high level of quinone reductase activity (174 to 288 nmol/min/mg protein) was measured in the luna moth, which performs well on juglone-containing diets (Lindroth, 1989).

We suggest that juglone is reduced in the intestinal system of *G. molesta*. Larval frass of *G. molesta* contained relatively high amounts of the trihydroxynaphthalene, but also some juglone, that may represent the compound derived directly from the diet. However, it is more likely that juglone is the product of a spontaneous reoxidation of 1,4,5-trihydroxynaphthalene in air (Hedin et al., 1980; Piskorski and Dorn, 2011). Various plant phenolic compounds are oxidized in the insect's gut (Summers and Felton, 1994; Barbehenn et al., 2003), but such reoxidation of trihydroxynaphthalene seems to be negligible, as indicated by the current work and two previous studies on juglone-tolerant lepidopterans (Lindroth, 1989; Piskorski and Dorn, 2011). Moreover, the absence of juglone in hemolymph and in larvae with emptied guts indicates that this compound is not sequestered by the insect. Hence, the mechanism of overcoming naphthoquinone-based plant defense in *G. molesta* may resemble that recently described for *C. pomonella* (Piskorski and Dorn, 2011).

Lack of infestation of Juglandaceae by the invasive *G. molesta* may have various reasons. First, the volatile profile of walnut (Bezemer and Mills, 2001; Witzgall et al., 2005; Casado et al., 2008) differs from that of peach, this herbivore's primary host (Natale et al., 2003) and from that of apple, its secondary host (Vallat and Dorn, 2005). Hence, the volatile blend emitted from walnut may fail to attract oriental fruit moth females during flight. Second, deterrent properties of naphthoquinones may prevent host acceptance, as shown for other insect species (Ferkovich and Norris, 1971; Faccoli et al., 2005). Third, the skin of walnut husks may pose a harder physical barrier than that of peach and other rosacean fruits, thus preventing penetration by neonate larvae. Finally, we cannot exclude that some infestations may have gone unnoticed, as has similarly been discussed for related internal fruit feeders (Chen and Dorn, 2009).

We do not know whether juglone influences other life-history traits in this herbivore, such as fertility, mating success, longevity, or flight capacity (Cisneros and Barnes, 1974; Keil et al., 2001; Moreau et al., 2006; Torriani et al., 2010). Lifetime fecundity may suffer in cases with reduced adult weight (Bezemer and Mills, 2001) as noted for the high, but not for the low juglone level reported from walnut husks. However, naphthoquinones can be antimicrobial (Clark et al., 1990; Inbaraj and Chignell, 2004) and can act as dietary antioxidants (Johnson and Felton, 2001; Rodríguez et al., 2007), so that by feeding on nutritional sources containing naphthoquinones, insect herbivores may compensate for some fitness losses, or even gain fitness benefits by being better protected against infections and strong oxidants.

It is commonly accepted that many herbivores can widen not only their geographic but also their host range (Dambroski et al., 2005; Myers et al., 2006; Erbout et al., 2009; Chen and Dorn, 2010; Piskorski et al., 2010). The European corn borer *Ostrinia nubilalis* (Lepidoptera: Pyralidae) successfully added maize to its original host range in spite of the toxic properties of hydroxamic acids from this plant, in particular 2,4-dihydroxy-7-methoxy-l,4-benzoxazin-3-one (DIMBOA) (Campos et al., 1988; Martel et al., 2003). Likewise, apple was initially reported only as a secondary, relatively unimportant host plant of the oriental fruit moth (Rothschild and Vickers, 1991), but its significance as a host is continuously increasing (Natale et al., 2004; Myers et al., 2006), indicating the potential of this insect to rapidly adapt to new hosts. Our results suggest that in future, *G. molesta* could include juglandacean plants into its host range, provided other factors are favorable.

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Introduction

In social insects, large aggregations of colonies from the same species occasionally occur where aggression among members from different colonies is absent. Such large colonies were most intensively studied in ants (supercolonies: Holway et al., 1998; Tsutsui et al., 2000; Drescher et al., 2007; supercolonies: Steiner et al., 2007; Helanterä, 2009). They are characterized by an absence of aggression of workers from different nests within supercolonies (Holway et al., 1998; Tsutsui et al., 2000; Giraud et al., 2002; Drescher et al., 2007) and frequent exchange of workers between nests (Pedersen et al., 2006). Supercolony formation may result from reduced genetic variation i.e. due to a population bottleneck (Tsutsui et al., 2000). It may also result from a reduced self-nonself recognition, i.e. due to a uniform composition of chemical compounds on their body surface (hitherto called "chemical profiles") (Giraud et al., 2002; Errard et al., 2005), or to low benefits in comparison to the costs of aggressive interactions (Steiner et al., 2007). In general, social insects distinguish between related and unrelated individuals based on interspecific and intercolonial differences in the number and amount of hydrocarbons (HCs) in their chemical profiles (Crozier and Dix, 1979; Getz and Chapman, 1987; Howard, 1993; Howard and Blomquist, 2005): Related individuals are chemically similar and hence tolerated, whereas unrelated individuals are chemically different and therefore aggressively attacked (Lacy and Sherman, 1983; Martin and Drijfhout, 2009).

Whereas the relationship between aggression, chemical profiles and relatedness has been frequently investigated in ants, nest aggregations of bee colonies have received comparatively little attention, although nest aggregations are common in solitary, semi-social and social bees (Wcislo, 1993; Kukuk et al., 2005; Thiele and Inouye, 2007; Gonzalez and Ospina, 2008; Haeseler, 2008; Timmermann and Kuhlmann, 2008). These nest aggregations frequently comprise unrelated individuals or colonies (Ward and Kukuk, 1998; Cameron et al., 2004).

There are multiple hypotheses on the causes of such nest aggregations (reviewed by Ward and Kukuk, 1998). One possibility may be a lack of suitable nest sites (Michener, 1974; Eltz et al., 2001) or that bees assess nest site quality by the presence of other colonies (Eltz et al., 2001). Aggregated nesting may also decrease the risk of individual nests to be subject to predators due to a 'selfish herd' effect, as has been shown in birds (Clark and Robertson, 1979) and in stenogastrine wasps (Landi et al., 2002). Alternatively, in bees, aggregated nests of unrelated colonies could facilitate mating between females and unrelated males, thereby significantly decreasing the risk of inbreeding (Cameron et al., 2004; Kukuk et al., 2005) which may be particularly costly in hymenoptera due to their sex determination mechanism (Cook and Crozier, 1995; Zayed and Packer, 2005).

In some tropical stingless bee species (Apidae, Meliponini), nests of several colonies can be heavily aggregated with up to eight colonies at the base of a single tree (Eltz et al., 2001; Cameron et al., 2004; Leonhardt et al., 2010b). In Southeast Asia, aggregated nests of unrelated colonies are most prominent in the stingless bee *Tetragonilla collina* (Roubik, 1996; Eltz et al., 2001; Cameron et al., 2004; Leonhardt et al., 2010b). Moreover, this species shows very low levels of aggression towards both conspecific and heterospecific individuals (Leonhardt and Blüthgen, 2009; Leonhardt et al., 2010b), although aggressive encounters between stingless bees can be commonly observed at floral resources and baits (Hubbell and Johnson, 1977; Nagamitsu and Inoue, 1997; Leonhardt and Blüthgen, 2009).

To better understand the mechanisms behind aggregated nesting in *T. collina*, we investigated whether behavioral responses of *T. collina* individuals can be predicted by variation in relatedness and/or chemical profiles. We tested for differences in behavioral responses in *T. collina* bees from (1) the same colony, (2) different colonies of the same aggregation and (3) distant aggregations. To examine whether behavioral responses correlated with genetic relatedness and/or chemical profiles, we analyzed and compared differences in both genetic relatedness and the composition of cuticular compounds (1) within colonies, (2) within aggregations and (3) between aggregations.

Methods

Study Sites, Aggregations and Bees

Sampling of specimens for genetical and chemical analyses as well as behavioral experiments were performed at two different field sites in Borneo (Malaysia), in August–November of 2007 and 2008. (1) The Danum Valley Conservation Area (DVC: Sabah, 4°55′ N 117°40′ E, 100 m asl) comprises one of the major remaining patches of Sabah's primary lowland dipterocarp rainforest (43 800 ha) (Marsh and Greer, 1992). (2) The Kabili Sepilok Reserve (KSR: Sabah, 5°54′ N, 118°04′ E, 20–120 m asl) covers an area of 4294 ha of coastal dipterocarp and mangrove forest (Fox, 1973) surrounded by oil palm plantations. Attached to KSR is the Rainforest discovery centre (RDC), a small (148.6 ha) education centre with secondary and planted vegetation. Both field sites have a

typical equatorial rainforest climate with a mean annual temperature of 26–30°C and a yearly rainfall of 2600–3000 mm (Fox, 1973; Sakai et al., 1997). Fifteen stingless bee species (species and genus names as in Moure, 1961) were recorded for DVC (Eltz, 2004), whereas 15 to 20 species can be found in KSR and RDC according to collections of specimens held by the Forestry Research Centre in Sepilok and our own studies (Leonhardt and Blüthgen, 2009).

Overall, we sampled 19 colonies from four nest aggregations (between 2 and 7 nests per aggregation) and two single, non-aggregated colonies (stcrf1 and sltc8) of *Tetragonilla collina* for genetical analyses. Note that stcrf1 nested in aggregation with another, morphologically similar species that was excluded from analyses due to strong differences in its chemistry and alleles for the genotyped loci, suggesting that it may represent a different species (Fig S1 and S2).

All colonies' nests were situated in or beneath tree trunks (14 nests) or in cavities in walls (seven nests). Aggregations were characterized by a high nest density with all nests having at least one neighboring nest less than 50 cm away and a maximum distance of ≤3 m from the most distant nest. Eight of these colonies (representing three different aggregations and one single colony) were tested for differences in behavioral responses towards colonies from the same or a distant aggregation (Table 1). Detailed chemical analyses were performed for overall twelve colonies from all four aggregations and one single colony (Table 1).

Bee Sampling and Behavioral Testing

Individual bees were caught at their nest entrances by putting a clean transparent plastic bag or container above the nest entrance tube. Behavioral experiments followed the protocols successfully applied to Bornean stingless bee species in Leonhardt et al. (2009) and Dworschak and Blüthgen (2010): A focal bee was directly transferred from the plastic bag or container to a clean arena (a plastic Petri dish inversely placed on a gauze that was tightened to a large plastic box) and left undisturbed for habituation. After 1 min, a dead bee (that was killed by freezing prior to the experiment) was inserted into the arena. We observed the behavioral responses of the focal living bee towards the dead bee ('alive against dead experiment') for a period of

Table 1 List of *T. collina* colonies sampled for genetical and chemical analyses and tested in the behavioral experiments

Nest ID	Location	Aggregation	r	C	Cr	Cg	N1	N2	N3
dtc1	DVC	D1	0.81±0.08	0.21±0.09	0.23±0.10	0.19±0.08	13	20 (10/10/0)	11
dtc2	DVC	D1	0.82±0.03	0.27±0.15	0.31±0.14	0.24±0.17	13	20 (10/10/0)	9
stcf1	KSR	S1	0.58±0.19	0.38±0.07	0.47±0.07	0.31±0.05	14	20 (10/10/0)	3
stcf2	KSR	S1	0.35±0.15	0.34±0.06	0.43±0.12	0.23±0.06	16	30 (10/10/10)	3
stcf3	KSR	S1	0.64±0.21	0.20±0.07	0.26±0.10	0.17±0.05	10	10 (10/0/0)	5
stcrf1	RDC	single	0.78±0.14	0.21±0.03	0.38±0.10	0.16±0.04	10	20 (10/0/10)	4
stcw1	RDC	S2	0.67±0.19	–	–	–	4	–	–
stcw2	RDC	S2	0.89±0.14	0.41±0.09	0.48±0.12	0.37±0.07	14	–	4
stcw3	RDC	S2	0.54±0.31	–	–	–	14	20 (10/10/0)	–
stcw4	RDC	S2	0.87±0.06	–	–	–	11	–	–
stcw5	RDC	S2	0.81±0.26	–	–	–	15	–	–
stcw6	RDC	S2	0.56±0.11	–	–	–	14	–	–
stcw7	RDC	S2	0.29±0.2	0.21±0.06	0.23±0.08	0.20±0.07	19	40 (10/10/20)	5
sltc1	KSR	S3	0.19±0.24	0.30±0.13	0.38±0.16	0.23±0.12	15	–	8
sltc2	KSR	S3	0.26±0.19	0.25±0.12	0.37±0.18	0.20±0.10	16	–	8
sltc3	KSR	S3	0.68±0.31	–	–	–	17	–	–
sltc4	KSR	S3	0.65±0.12	0.26±0.16	0.33±0.23	0.23±0.13	15	–	8
sltc5	KSR	S3	0.86±0.16	–	–	–	10	–	–
sltc6	KSR	S3	0.13±0.28	0.26±0.10	0.32±0.13	0.22±0.09	10	–	8
sltc7	KSR	S3	0.68±0.08	–	–	–	11	–	–
sltc8	KSR	single	0.81±0.25	–	–	–	10	–	–

Within-colony relatedness [± SE] (r), chemical variation (equivalent to mean Bray-Curtis distances [± SD]) for all compounds (C), only resin-derived compounds (Cr) and only non-resin derived compounds (Cg) as well as the number of individuals used for the microsatellite analysis (N1), tested in behavioral experiments (N2: numbers in brackets give the number of bees tested in control/intra-aggregation/inter-aggregation trials) and used for chemical analyses (N3) are given

3 min, assigning all behavioral responses observed towards the dead bee to one of the following three response levels: '1' neutral response (investigation of dead bee with antennae, mandibles closed), '2' slight aggression (open mandibles), and '3' high aggression (biting in extremities, biting off body parts, dragging dead bee around arena). We calculated the aggression level (A_i) for each focal bee individual i as described in Leonhardt et al. (2010b): $A_i = (L2 + L3)/(L1 + L2 + L3)$, where L1, L2, and L3 are the number of behavioral responses of each aggression level. We tested for differences in aggression as well as the overall number of responses between control trials, inter-colonial trials with associated colonies and inter-colonial trials with non-associated colonies using Kruskal Wallis tests.

Both focal and dead bees were tested only once. Ten replicates were performed per inter-colonial trial and ten per intra-colonial trial (control trials). Overall, 180 bees (eight colonies) were tested (four bees that died during the test trial were excluded from the analysis, Table 1).

Microsatellite Analysis

Individual bees were collected from nest entrances of each of 21 colonies, killed in a freezer and directly transferred into 99% ethanol. In total 273 bees were analyzed. Between 4 and 19 individuals were analyzed per colony (mean ± SD 12.9±3.33 bees per colony). Genomic DNA was extracted from abdomen tissue of individual workers using Puregene ® DNA Purification Kit (Gentra Systems) according to the manufacturer's recommendations. DNA pellets were resuspended in 50 µl low TE and stored at −20°C.

Initially five microsatellite loci were used, that were developed for *Tetragonula carbonaria* (Green et al., 2001): Tc1.20, Tc3.155, Tc3.56, Tc7.13 and Tc4.287. The locus Tc1.20 gave poor amplification results and was later omitted from the analysis. PCR amplification was performed in an Eppendorf Mastercycler® or Biometra T1 Thermocycler®, in a total reaction volume of 12.5 µl containing approximately 10 ng of template DNA, 1x PCR-buffer, 1.1 mM $MgCl_2$, 160 µM dNTPs, 2.5 µM of each primer (forward primer labeled with fluorescent IR-700 or IR-800dye) and 0.5U of *Taq* DNA polymerase (*MolTaq*®, Molzym GmbH). Cycle parameters were as follows: 3 min at 94°C, followed by 30 cycles of 94°C for 30 sec, annealing step 30 sec, 72°C for 30 sec, and a final extension of 8 min at 72°C. Annealing temperatures were as follows: Tc3.155: 67°C; Tc3.56: 64.5°C; Tc4.287: 52°C; and Tc7.13: 58°C. PCR-products were diluted between 1:5 and 1:30 and analyzed on a LICOR® 4300 DNA Analyzer.

To test for deviation from Hardy-Weinberg-Equilibrium (HWE) and genotypic linkage disequilibrium (LD) we generated four datasets with one randomly chosen worker from each colony. HWE and LD were tested using Genepop On The Web (Raymond and Rousset, 1995: http://genepop.curtin.edu.au/). Relatedness within and between colonies and aggregations was calculated using Relatedness 5.0.8 (Queller and Goodnight, 1989), including all individuals as reference population and colonies weighted equally. Standard errors were obtained by jack-knifing over loci.

The partitioning of genetic variance within the population was determined by a hierarchical AMOVA based on F_{ST} as implemented in ARLEQUIN 3.1 (Excoffier et al., 2006). We performed a three-level hierarchical AMOVA with the nested levels: whole population variance, within-aggregation variance and within-nest variance.

Chemical Analysis

Between 3 and 11 individuals were sampled from overall 12 colonies and separately extracted in hexane for 2 min, resulting in 75 individual *T. collina* extracts. We analyzed these extracts using a Hewlett Packard HP 6890 Series Gas Chromatographic System coupled to a Hewlett Packard HP 5973 Mass Selective Detector (Agilent Technologies, Böblingen, Germany). The GC was equipped with a J & W, DB-5 fused silica capillary column (30 m×0.25 mm ID; df=0.25 µm; J & W, Folsom, CA, USA). Temperature was programmed from 60°C to 300°C with a 5°C/min heating rate and was held for 10 min at 300°C. Helium was used as carrier gas (constant flow of 1 ml/min). Injection was carried out at 250°C in the splitless mode for 1 min. Electron impact mass spectra (EI-MS) were recorded at an ionization voltage of 70 eV and a source temperature of 230°C. We used the Windows version of the ChemStation software package (Agilent Technologies, Böblingen, Germany) for data acquisition. Compounds were characterized as described in Leonhardt et al (2009), by comparing their mass spectra and retention indices with mass spectra and retention indices from three commercially available libraries (Wiley 275, NIST 98 and Adams EO library 2205), and by comparing them to synthetic standards (Sigma-Aldrich, Munich, Germany) if standards were available.

To test for differences in the cuticular chemical profiles between individual *T. collina* bees from (1) different colonies, (2) different aggregations, and (3) different regions, we performed permutation tests (adonis, R-package vegan 1.15, 10000 runs) based on relative peak areas. Relative peak areas were calculated by dividing each peak's integrated area by the total integrated peak area. To account for potential differences between non-resin-derived CHCs and resin-derived compounds, we performed separate Adonis tests confined to these two groups of compounds. Graphs for visual presentation were produced by two-dimensional NMDS (non-metric dimensional scaling). Bray-Curtis dissimilarities based on the proportions of all compounds that accounted for more than 0.5% of the

total peak area in at least one sample (start configuration: PCoA, 1000 iterations) were used for NMDS and Adonis tests. Overall, 82 compounds were used for the analysis, 47 of which were most likely derived from plant resins (environmentally derived). A detailed list of compounds is given in Table S2.

To test whether related individuals had more similar chemical profiles than unrelated individuals, we performed a Mantel test based on Pearson's product moment correlation using relatedness and chemical distances among colonies.

Results

Behavioral Tests

Only 21 out of 176 *T. collina* bees tested showed little aggression (open mandibles) towards dead bees from either their own colony (13% of bees tested in control trials were slightly aggressive), their own aggregation (15%) or a distant aggregation (5%). The large majority of bees tested responded non-aggressively towards dead bees no matter whether they were taken from their own or a foreign colony. Hence the number of aggressive responses towards dead bees did not differ for bees of the same colony (control trials: mean aggression [± SD] A_i=0.10±0.28), bees of different colonies but the same aggregation (mean A_i=0.12± 0.30), or bees of distant aggregations (mean A_i=0.04±0.18; Kruskal Wallis test: H=2.07, p=0.35). They also showed no differences in the overall number of responses (aggressive and non-aggressive ones) towards dead bees (Kruskal Wallis test: H=1.95, p=0.38).

Relatedness Within and Between T. collina Colonies

The 271 bees analyzed were all successfully genotyped for at least three of the four loci with good amplification results. Locus Tc7.13 showed the highest number of failed amplifications with only 194 individuals being successfully genotyped. For the other three loci, at least 235 individuals were successfully genotyped. Failed amplifications were not concentrated on particular colonies but scattered among colonies. Number of alleles per locus ranged from 3 (Tc7.13) to 5 (loci Tc3.155 and Tc3.56) (for details see Table 2). The four loci showed no significant linkage disequilibrium.

Out of the 16 tests performed (4 loci x 4 datasets containing a single individual per colony), 3 showed significant deviation from HWE (heterozygote deficiency). In two out of the four datasets, the locus Tc4.287 deviated significantly from HWE and Tc7.13 in one (Fisher's exact test; p<0.05).

Table 2 Characteristics of microsatellite loci for *T. collina* workers

Locus	N	A	H_O	H_E
Tc3.155	235	5	0.581	0.489
Tc3.56	247	5	0.488	0.612
Tc4.287	249	3	0.487	0.541
Tc7.13	194	4	0.443	0.560

N number of individuals genotyped; *A* number of alleles; H_O observed heterozygosity and H_E expected heterozygosity, based on a dataset containing four randomly chosen workers per colony

(a) Relatedness within colonies:

Within-colony regression relatedness of the 21 colonies sampled ranged from 0.133 to 0.888 (mean ± SD: 0.61±0.24) and was on average significantly lower than 0.75, the expected relatedness among full sisters in monogynous colonies (one-sample t-test against 0.75, p=0.015, T=−2.65, df=20). Five of the 21 colonies showed within-colony relatedness values lower than 0.353 (Table 1), thus deviating from the expected within-colony relatedness of 0.75 where all workers are full sisters and derived of a single monandrous queen.

(b) Relatedness within aggregations:

Aggregations comprised both related and unrelated colonies (Table S1). Within-aggregation relatedness was highest in aggregation D1 in DVC with the two colonies being closely related (r=0.71±0.11), whereas all aggregations in KSR and RDC (S1 to S3) showed variable within aggregation relatedness (average relatedness of colonies in S1: r=0.16±0.42 comprising 3 nests; S2: r=0.07±0.06 comprising 7 nests; S3: r=0.14±0.07 comprising 7 nests).

(c) AMOVA results: partitioning of genetic variance:

The AMOVA revealed that a significant proportion of the genetic variance was explained by within-nest variation (67.7%; p<0.0001) as well as by variation among nests within aggregations (32.6%; p<0.0001). In contrast, variance between aggregations (within-population variance) did not contribute significantly to the total genetic variance (−0.2%; p=0.48 n.s.).

Chemical Profiles

The twelve *T. collina* colonies analyzed generally varied in their chemical profiles (Fig. 1, Adonis: R^2=0.50, p<0.001) with chemical differences being more pronounced between (mean Bray-Curtis distance [± SD]: 0.45±0.16, number of distance pairs N=5414) than within (0.31±0.15, N=1726) colonies. The three aggregations also had different chemical profiles (Fig. 1, R^2=0.36, p<0.001). However, when colonies were directly compared to one another, only 26

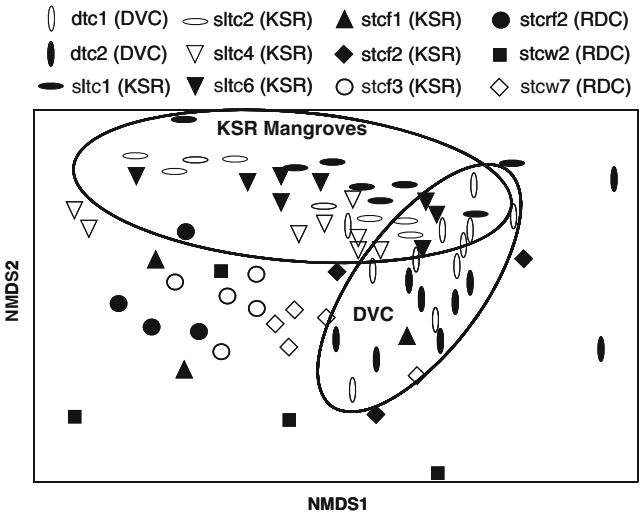


Fig. 1 Similarity in chemical profiles of surface compounds from 12 *Tetragonilla collina* colonies of five nest aggregations in Borneo (ordination figure based on Bray-Curtis dissimilarities, stress value= 13.87). Different symbols represent different colonies (each symbol represents one individual bee): *Circled* colonies mark two colonies from an aggregation in DVC (black and white upright ellipses) and four colonies from an aggregation found close to the coast in KSR (black and white ellipses, *black and white triangles* upside down). The remaining colonies stem from one aggregation in the interior forest of KSR (*black diamond*, *black triangle* and *white circle*), and one aggregation (*white diamond* and *black square*) as well as one single colony (*black circle*) at the RDC

out of 66 colony pairs could be distinguished (with *p*-values<0.001, which was the required significance level according to Bonferroni correction for multiple testing).

As expected, non-resin derived compounds more clearly separated both colonies and aggregations (colonies: R^2=0.54, p<0.001; aggregations: R^2=0.40, p<0.001) than did resin-derived compounds (colonies: R^2=0.44, p<0.001; aggregations: R^2=0.31, p<0.001). Resin-derived compounds further showed an on average higher chemical variation within colonies (mean Bray Curtis distance: 0.35±0.08) than did non-resin derived compounds (mean Bray Curtis distance: 0.27±0.07; Table 1).

Chemical similarity among *T. collina* colonies did not correlate with degrees of relatedness, neither for all compounds (Mantel test: r=−0.13, p=0.84), nor for resin-derived (r=−0.17, p=0.88) or non-resin derived compounds (r=−0.10, p=0.72).

Discussion

Colonies of the stingless bee *Tetragonilla collina* frequently nest in aggregation with up to seven other conspecific colonies in our study area, but did not show any aggressive behavior among each other. Likewise, aggression was absent between colonies of different aggregations. This atypically peaceful behavior contrasts with other studies on stingless bees that found relatively strong aggression between con- or heterospecific non-nestmate individuals (Johnson and Hubbell, 1974; Hubbell and Johnson, 1977, 1978; Howard, 1985; Inoue et al., 1999; Kirchner and Friebe, 1999; Nunes et al., 2008; Leonhardt and Blüthgen, 2009).

In contrast to honeybees where new colonies are established by old queens while young queens remain in their mother nests, new stingless bee colonies are founded by young queens (Inoue et al., 1984; Roubik, 1989). These young queens frequently build their nests in close proximity to their mother colony and the two nests exchange workers and resources until the new colony finally becomes independent (Inoue et al., 1984). *Tetragonilla collina* nest aggregations can therefore contain a mother and several daughter colonies which may explain why five colony pairs within the aggregations investigated in our study were more closely related (> 0.40) than colonies of distant aggregations and why chemical profiles were more similar within than among aggregations. However, 39 out of 52 aggregated colony pairs were only little related (< 0.25). Likewise, Cameron and colleagues (Cameron et al., 2004) found no or only little relatedness among aggregated *T. collina* colonies in Thailand, indicating that aggregations may include both related and unrelated colonies. Hence, relatedness among colonies may account for the lack of aggression in some behavioral assays, such as in the pairing of colony stcf1 and stcf3 (with relatedness being 0.23±0.29), but not in assays conducted with bees from unrelated colonies (see Table S1).

Whether the *T. collina* population sampled in this study generally shows only little genetic variability in the markers used or whether this low level of variability is due to high population viscosity can currently not be fully resolved. Nonetheless, different degrees of relatedness did not translate into different degrees of aggressiveness in this species of stingless bee. Within colony relatedness did further not correlate with chemical similarity, neither for environmentally derived nor for genetically determined compounds: Two out of five colonies with low within-colony relatedness showed very little chemical variation (colonies stcw7 and sltc2), but one colony (stcw2) with high within-colony relatedness had a strongly diverging chemical profile (Table 1). Chemical variation among individuals of the same colony was comparatively large in five of our 12 colonies (Fig. 1). However, overall, all colonies, and particularly those within aggregations, had qualitatively and quantitatively similar chemical profiles, and 40 out of 66 colony pairs could chemically not be distinguished.

Lack of statistical discrimination between chemical profiles does however not imply a lack of recognition. It is unknown whether stingless bees use the entire bouquet of

chemical compounds on their body surface to recognize colony members or whether they rely on a subset of compounds, as suggested for other social insects. For instance, ants also have a variety of hydrocarbons on their body surfaces, including saturated- and unsaturated ones linear ones as well as methyl-branched ones (reviewed by Martin and Drijfhout, 2009). However, in two *Formic*a ant species, unsaturated (n-alkenes) or branched hydrocarbons were found to be more important for the recognition of colony members (nestmates) than linear n-alkanes (Akino et al., 2004; Martin et al., 2008). Likewise, in the honeybee *Apis mellifera*, n-alkenes and fatty acids seemed to be most important for nestmate recognition (Châline et al., 2005; Dani et al., 2005). The neotropical stingless bee *Trigona fulviventris* also used fatty acids to differentiate between nestmates and non-nestmates, but n-alkanes and floral oils further affected their recognition ability (Buchwald and Breed, 2005). To our knowledge, there are no other studies that have investigated nestmate recognition cues in stingless bees (let alone in any paleotropical stingless bee species). It is thus possible, yet remains to be shown, that stingless bees can obtain sufficient information about another bee's colony identity from only a subset of surface compounds or from compounds not detected by the GC-MS (e.g. proteinaceous compounds), which is likely to be overlooked by our chemometrical analysis. More studies on compounds or compound groups used as recognition cues by stingless bees are therefore needed.

Besides cuticular hydrocarbons (CHCs), the chemical profile of *T. collina* comprises large amounts of terpenoid compounds (Leonhardt et al., 2009, 2010a). By comparing the bees' profiles with compounds of plant resins, we found that these terpenoids were derived from resins (Leonhardt et al., 2011) which the bees collect for nest construction, maintenance and defense (Leonhardt and Blüthgen, 2009; Leonhardt et al., 2009). The composition of resin-derived terpenoids is highly species-specific, rendering mere contamination of the bees' surfaces by resin unlikely (Leonhardt et al., 2009). Moreover, we found that resin-derived terpenoids show a species-specific distribution even on the bees' wings (Leonhardt et al., 2009) – the insect body part considered least prone to contamination (McDaniel et al., 1984). The terpenes may (at least) partly account for the lack of correlation between genetic relatedness and chemical similarity. They may further increase the chemical overlap between different *T. collina* colonies, because chemical distinction was less pronounced (R^2=0.44) and chemical variation was larger (mean Bray-Curtis distance [± SD]: 0.35±0.08) for terpenes than for non-resin-derived CHCs (R^2=0.54, mean Bray-Curtis distance: 0.27±0.07). Moreover, the addition of environmentally derived compounds increases the diversity of chemical compounds in the bees' surface profiles and thus potentially also cue diversity. It was suggested that increased cue diversity can cause cue overlap amongst colonies and thus a decrease in the accuracy or even a total lack of nestmate recognition (Peeters, 1988; Satoh and Hirota, 2005; Martin et al., 2009). Whether terpenes do actually play a role in the nestmate recognition system of stingless bees is as yet unknown. Sesquiterpenes – a group of terpenes that makes up for the majority of terpenes on the body surface of *T. collina* – were found to reduce aggression in another stingless bee species that lacks sesquiterpenes itself (Leonhardt et al., 2010b). Consequently, environmentally derived terpenes in the chemical profiles of stingless bees may or may not be directly involved in nestmate recognition. If not directly involved, some terpenes may still impair the bees' ability to distinguish between related and unrelated individuals and thus (at least partly, albeit not exclusively) explain the lack of aggression in *T. collina*.

One would expect that a lack of aggression in an insect colony as well as aggregated nesting of multiple colonies should favor social parasitism by either foreign queens or foreign workers ("floaters" or "drifters": Lopez-Vaamonde et al., 2004; Sumner et al., 2007). Unless they are hindered by resident bees, foreign queens may take over nests and produce own offspring, while foreign workers may produce males (brood parasitism', reviewed by Beekman and Oldroyd, 2008) or simply steal food reserves (cleptoparasitism: Cardinal et al., 2010) which frequently occurs in stingless bees (Roubik, 1989, 2006). Social parasitism by egg-laying workers entering unrelated colonies has been found in the stingless bee *Melipona scutellaris* (Alves et al., 2009). Alternatively, queen supersedure within colonies may explain the reduced within-colony relatedness that we observed in some colonies. Queen supersedure was also found quite frequently in stingless bees (Paxton et al., 2003; Alves et al., 2009). However, only five out of our 19 colonies had within-colony relatedness values below 0.4 (indicating the presence of unrelated individuals), whereas eight colonies even had relatedness values above 0.7, matching predictions for full sisters in colonies with a single-mated queen. Hence, foreign individuals may occur in *T. collina* colonies but do not appear to be common.

We cannot exclude that aggression towards foreign individuals occurred in the inner nest parts of *T. collina* colonies, as we observed bees only outside their nests and in a relatively artificial arena environment. However, our aggression assays were successfully applied to several Bornean stingless bee species (testing encounters between both two alive bees and one alive and one dead bee) and revealed strong aggression for inter-colonial as well as inter-specific encounters (Dworschak and Blüthgen, 2010; Leonhardt et al., 2010b). Notably, *T. collina* did not show any aggression towards other stingless bee species neither

when tested in behavioral assays (Leonhardt et al., 2010b) nor when observed in the field (Leonhardt and Blüthgen, 2009). *T. collina* can thus clearly be considered less aggressive than other Bornean stingless bee species. The causes for its unusual 'peacefulness' remain unclear.

Reduced aggression or even a lack of aggression does however not necessarily correlate with reduced or lack of recognition (Chapuisat et al., 2005; Steiner et al., 2007; Martin et al., 2009). It can also stem from high costs of erroneously rejecting related individuals, i.e. in situations of reduced between-colony competition (Reeve, 1989; Downs and Ratnieks, 2000), low costs of accepting foreign workers or to save resources better used for colony growth and reproduction (Holway et al., 1998), because aggression is costly (Reeve, 1989). Thus, *T. collin*a may still be able to distinguish between individuals of its own and a foreign colony, as has, for instance, be shown in *Formica paralugubris*: these ants successfully discriminated between nestmates and non-nestmates (as indicated by significantly higher rates of trophallaxis exchanged with nestmates than with non-nestmates), but showed little to no aggression towards unrelated individuals (Chapuisat et al., 2005). *Lasius austriacus* ants were equally non-aggressive towards unrelated individuals, but performed significantly longer antennation bouts with unrelated than with related ants (Steiner et al., 2007). In contrast to these authors, we did however not find a difference in the overall number of behavioral responses (including aggressive and non-aggressive ones).

Conclusions

Relatedness did not generally explain the lack of aggression between *Tetragonilla collina* nests within aggregations, as colonies found in aggregation could be either closely or distantly related. Moreover, the composition of compounds on the cuticle of *T. collina* bees was no more similar for related than for unrelated individuals, nor did low within-colony relatedness correlate with a larger chemical variation. The observed chemical divergence was mainly due to environmentally derived resin-compounds which are found in the chemical profile of *T. collina* in addition to genetical determined cuticular hydrocarbons. The presence of these compounds may partly explain the lack of aggression in *T. collina*, as it may result in decreased recognition acuity due to either higher cue diversity or to impairing of recognition.

It remains unclear why most of the aggregated *T. collina* colonies studied showed relatively high within-colony relatedness (partly even matching predictions for colonies with a single-mated queen), although both lack of aggression and aggregated nesting should favor social parasitism by unrelated workers or queens.

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males from approaching. These studies suggest that inhibition of the approach of males to females by use of pheromones may function as: a) a pre-mating mechanism, preventing the approach of suitors during a courtship encounter; and b) a post-mating mechanism, preventing subsequent mating by the female, thereby benefiting sperm precedence of the last male that copulated (the most recent male to mate prior to oviposition is the one most likely to fertilize the most eggs; Andersson et al., 2003).

Most studies on the volatiles released by male Lepidoptera during courtship, however, have shown that such compounds attract females or cause females to become quiescent, thus facilitating copulation (Birch et al., 1989). In many species, HP volatiles have a clear effect on female behavior and influence the overall mating success of a courting male (Fitzpatrick and McNeil, 1988; Hillier and Vickers, 2004). In *G. molesta*, males 'flash' abdominal HPs directly in front of females that initiates short-range attraction (Baker and Cardé, 1979; Baker et al., 1981; Baker 1983). HP volatiles more frequently function as arrestants of female behavior, subduing activity and thereby increasing the probability of male mating success (Birch et al., 1990).

HP ablation studies (effectively, volatile removal) have shown the necessity of HP volatiles during courtship in several species. In *Pseudaletia unipuncta* Haworth, HP ablation caused a decrease in female acceptance of courting males (Fitzpatrick and McNeil, 1988). Female antennectomy or male HP ablation also significantly lowered mating success in courtship by the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae; Royer and McNeil, 1992). In *H. virescens*, proper HP display by males is critical for successful mating (Cibrian-Tovar and Mitchell, 1991).

The involvement of HP pheromones in courtship has been hypothesized as a mechanism of intersexual selection through female mate choice (Baker and Cardé, 1979). The elaboration of male-specific structures, and the diversity of male courtship volatiles observed across taxa, suggest that HP volatile composition may communicate information that is species-specific (Hillier and Vickers, 2004). Male pheromones in courtship behavior are thought to be especially important between congeneric, sympatric species, particularly if the female sex pheromone composition is similar (Birch et al., 1990). Under such circumstances, there should be greater selective pressure to prevent interspecific mating.

Previous research has demonstrated that male *H. virescens* and *H. subflexa* display HPs during courtship with conspecific females (Laster, 1972; Teal and Tumlinson, 1997). These species can hybridize under laboratory conditions, though this has a direct fitness cost, producing sterile F_1 males (Laster, 1972; Proshold and LaChance, 1974). Several studies have described the courtship behavior of *H. virescens* and *H. subflexa*, along with F_1 hybrids and backcrosses to each species (Laster, 1972; Cibrian-Tovar and Mitchell, 1991). In one of the first studies to examine hybridization in these species, Laster (1972) found *H. virescens* males and *H. subflexa* females readily mated, but *H. subflexa* males and *H. virescens* females did not. Teal and Tumlinson (1997) hybridized males and females in both directions, using synthetic female sex pheromone to increase male courtship activity to females of the other species. HP display appears important for males of either species to mate successfully with *H. virescens* females, but not for *H. subflexa* females (Cibrian-Tovar and Mitchell, 1991). Furthermore, *H. subflexa* males have smaller HP structures relative to other heliothine species [such as *H. virescens* or *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae)], and produce smaller quantities of pheromone (Teal and Oostendorp, 1993, 1995).

In *H. virescens*, HP volatiles facilitate mating with females capable of discriminating between HP mixtures of closely related species (Hillier and Vickers, 2004). HP composition in both *H. virescens* and *H. subflexa* is primarily a blend of saturated 14–18 carbon-chain alcohols, acetate esters, and carboxylic acids (Teal and Tumlinson 1989, 1997; Teal and Oostendorp 1995). Antennectomized *H. virescens* females and HP-ablated *H. virescens* males mate less frequently than sham-operated counterparts. Interestingly, female acceptance of HP-ablated males may be restored in the presence of *H. virescens* HP extract, but not with extracts from the congeneric *H. subflexa* or *H. zea*. This suggests that HP volatiles function as a means of pre-zygotic reproductive isolation in this species.

Heliothis virescens and *H. subflexa* occur sympatrically, although there is little evidence that hybridization occurs naturally (Heath et al. 1990; Teal and Oostendorp 1995). This has largely been attributed to differential attraction and repulsion by males to components of the respective female sex pheromones. Groot et al. (2006) found in field trials that cross-attraction between these species is low (i.e., a very small percentage of males were attracted to females of the other species). During incidences in which individuals of the two species come into contact by chance, or through response to an inappropriate blend, it is possible that secondary characteristics such as HPs play a role for specific discrimination. The role of HP volatiles in preventing interspecific mating in sympatric species is not well understood.

Preliminary observations of *H. subflexa* mating indicated that courtship occurs more quickly than in *H. virescens* (personal observation). The reduced prominence of *H. subflexa* HPs, along with observations that *H. subflexa* females might not require HP display for mate acceptance, suggested that these structures are of diminished importance in *H. subflexa* courtship.

There were two major objectives of this study. The first was to determine the importance of HP volatiles in mating of *H. subflexa*, and the second was to assess the effects of male HP extracts on interspecific mating attempts by *H. virescens* and *H. subflexa*.

Methods and Materials

Insects

Virgin female and male moths from the *H. virescens* and *H. subflexa* colonies at the University of Utah were used for behavioral assays and HP extraction. *Heliothis virescens* larvae were reared on a pinto bean diet, while *H. subflexa* larvae were reared on a corn-soy diet, until pupation (Shorey and Hale, 1965). Pupae were separated by sex and placed in an environmental chamber (25°C, 60% relative humidity, on a reversed 14L:10D light cycle). Male and female pupae of each species were placed in separate 250-ml paper cups filled with moistened vermiculite, in different chambers, in order to prevent pre-mating exposure of either sex to the conspecific pheromones. Upon eclosion, moths were provided with sugar water (1:5 *w*/*v*) in a 30 ml cup with a paper towel wick. Males and females of each species (3–5 d-old) were selected for use in behavioral assays. Moths were placed in a darkened experimental room to acclimate for 1 hr before experimentation. Before and during experiments, containers with each sex were kept separated under a ventilated airflow (0.40 m.sec^{-1}, generated via a laboratory vacuum line) to minimize pheromone pre-exposure. Assays were conducted between the 3rd and 5th hours of the scotophase.

Volatiles

Preliminary observations showed that the number of mating attempts by males to a female of the other species was low. To facilitate male courtship, and to increase the number of copulation attempts, an artificial lure resembling the conspecific female sex pheromone was presented during interspecific mating trials (Teal and Tumlinson, 1997). Female sex pheromone sources used in interspecific mating trials were prepared by using stock solutions of synthetic female sex pheromone compounds from Bedoukian Research Inc. (Danbury, CT, USA). These solutions were diluted as a decade series in hexane (10 ng/μl–100 μg/μl) and stored at −20°C until used. Samples of stock solutions were confirmed as >95% purity by analysis with a Shimadzu GC 17A gas chromatograph, equipped with a 30 m×0.25 mm ID DB-5 capillary column. For the *H. virescens* female odor source, a mixture of (*Z*)-11-hexadecenal (*Z*11-16:Ald; 50 μg) and (*Z*)-9-tetradecenal (*Z*9-14:Ald; 2.5 μg) on a red rubber septum was used (Tumlinson et al., 1975; Tingle et al., 1978; Klun et al., 1980; Pope et al., 1982; Vetter and Baker, 1983; Teal et al., 1986). The *H. subflexa* female odor source consisted of *Z*11-16:Ald (50 μg), (*Z*)-9-hexadecenal (*Z*9-16:Ald; 25 μg) and (*Z*)-11-hexadecenol (*Z*11-16:OH; 5 μg) in a ratio of 10:5:1, respectively. This blend elicits upwind flight by *H. subflexa* males (Klun et al., 1982; Vickers, 2002).

HP extracts from male *H. virescens* and *H. subflexa* were produced by excising the HPs of 20 males into 1.0 ml hexane. For behavioral assays, one male equivalent (1ME; 50 μl of extract) was applied to a 1 cm diam. filter paper disc, held by an alligator clip attached to a 23 cm wire post. Hexane was allowed to evaporate from the filter paper prior to behavioral testing, and the post was rinsed with acetone between trials to prevent cross-contamination.

Behavioral Assays

Observations of moth mating behavior were conducted as previously described (Hillier and Vickers, 2004). Briefly, experiments were conducted in a dark room, illuminated by a single red incandescent bulb dimmed by a rheostat. Room temperature was 20.2–24.0°C. Mating assays were conducted in a circular chamber, consisting of two 17.5 cm diam Plexiglas plates, above and below a 6 cm tall wire mesh wall (Hillier and Vickers, 2004). The chamber could be rotated around a screw at the center of the base plate, and was mounted to the laboratory bench. An intake duct, positioned 2 cm from the chamber, maintained a suction airflow of 0.20–0.40 m.sec^{-1}. Moth behavior was recorded with a video camera (Panasonic© WV-BP330 CCTV) set up on the opposite side from the intake duct. Rotation of the chamber ensured that female moths were upwind from male moths at the commencement of courtship, and that the moths could be kept within the plane of focus of the camera.

Individual female moths were observed prior to all assays for calling behavior. Only those females that exhibited a stable calling posture (stationary, wings vibrating with ovipositor exposed) were used. The chamber was turned to position females at the most upwind portion; single males were placed in the downwind portion. Behavior was observed for 5 min. If moths engaged in behavioral interactions during this 5 min. period, the trial continued for an additional 5 min. If no interaction occurred, the moths were removed. Female behaviors were recorded in response to specific male courtship bouts, along with the number of mating attempts and copulation success or failure. A successful mating was defined as a clasp by a male suitor lasting for at least 1 min. during the 5 min period of interaction. Behaviors observed were: moving away (female moved more than 40 mm away from source

or suitor), flying away (also part of the 'move away' behavior, but more vigorous movement, involving flight to the opposite side of the chamber), wing fanning, ovipositor extension, abdominal extension, abdominal curling, clasping and false clasping (for a more complete description of these behaviors see Hillier and Vickers, 2004). Abdominal extension, abdominal curling, clasping, and false clasping are associated with successful mating attempts.

HPs were ablated by applying gentle pressure to the abdomen, and then trimming the hairs with iridectomy scissors. Sham-operated males were squeezed to expose HPs, but no hairs were removed. All sham-operations and ablations were conducted before the onset of the scotophase on the same day as the insects were tested. One hour before experimentation, the moths were placed in the experimental room for acclimation to light conditions. Two experiments were conducted:

Experiment 1: Effect of HP Ablation and Replacement on *H. subflexa* Mating

To determine whether or not HP volatiles are important in courtship and mate acceptance in *H. subflexa*, females were paired with either HP-ablated males, with or without *H. subflexa* HP extract, or with sham-operated male *H. subflexa*. The odor source was presented only in behavioral assays when an active courtship bout was exhibited by the male (i.e., only during the period when the HP-ablated male would normally be releasing HP volatiles). During these situations, the odor source was held 1–2 cm upwind from the female. The control for this experiment was a filter paper disc with hexane applied to it, and presented similarly.

Experiments 2 (a and b): HP Volatiles and Interspecific Mating

To determine whether HP volatiles influence the success of interspecific mating or not, two experiments were conducted: (a) with *H. subflexa* males and *H. virescens* females, and (b) with *H. virescens* males and *H. subflexa* females. In each experiment, female behavior and mating success were recorded in response to the following: a sham-operated interspecific male, a sham-operated interspecific male with conspecific HP extract, an HP-ablated interspecific male with conspecific HP extract, and an HP-ablated interspecific male with a hexane source. Pairs of male and female *H. virescens* and male and female *H. subflexa* also were tested as a reference for behavioral and mating frequency in each experiment.

Data Analysis

Frequencies of mating attempts, moving away, flying away, wing fanning, ovipositor extension, abdominal extension, abdominal curling, clasping, and false clasping were calculated for each experiment, with a *chi-square* test of independence and Yates correction ($P>0.05$) being used to determine differences in the frequencies of the behaviors. Differences in the mean number of mating attempts were analyzed using a one-way analysis of variance. All analyses were conducted with STATISTICA© 6.0 (Statsoft, Tulsa, OK, USA; Statsoft Inc., 2007).

Results

Experiment 1: Do HP Volatiles Increase Mating Success for *H. subflexa* Males?

Frequency of successful mating by male *H. subflexa* with conspecific females was affected by HP ablation. Mating by HP-ablated male *H. subflexa* was reduced ($P<0.001$) relative to that of sham-operated males, either with or without a hexane blank source (Table 1). When a filter paper loaded with 1ME of *H. subflexa* HP extract was presented along with HP-ablated males, the frequency of successful mating increased, and was greater than that of HP-ablated males in the presence of a hexane source. This suggests a key role for male-produced volatile signals in *H. subflexa* courtship.

Consistent differences were found in the behavioral responses of females among treatments (Fig. 1). Successful clasping was reduced in the HP-ablated male treatments compared to all other treatments. The frequency of false clasping was increased in both sham-operated male + hexane and HP-ablated male + hexane treatments. No differences ($P>0.05$; data not shown) were found in the number of mating attempts among males from any of the treatments.

Experiment 2: Do HP Volatiles Increase Interspecific Mating Success?

H. subflexa Males and H. virescens Females

For trials between male *H. subflexa* and female *H. virescens*, several differences were observed among HP treatments (Table 1). Control (intraspecific) pairs mated successfully 88.0% and 100.0% of the time, for *H. virescens* and *H. subflexa*, respectively. The frequency of mate acceptance between sham-operated male *H. subflexa* and female *H. virescens* was lower ($P<0.001$) than that of *H. subflexa* pairs (Table 1). Presentation of a hexane-loaded filter paper disc during the observations did not affect mating acceptances for the intraspecific pair compared to the sham-operated or HP-ablated male treatments (Table 1). Presentation of *H. virescens* HP extract (1ME) during courtship resulted in an increase in mate acceptance only in

Table 1 Frequency of mate acceptance for: (a) Female *Heliothis subflexa* and conspecific males with sham-operated or ablated hair pencils (HPs), and a source loaded with either *H. subflexa* HP extract or hexane; (b) Female *H. virescens* and *H. subflexa* males with sham-operated or ablated HPs, and a source loaded with either *H. virescens* HP extract or hexane; and (c) Female *H. subflexa* and *H. virescens* males with sham-operated or ablated HPs, and a source loaded with either *H. subflexa* HP extract or hexane

Mate Pairing and Treatment	*N*	Frequency of mate acceptance (±SEM)[a]
(a) Conspecific mating - *H. subflexa* female with: (X^2=26.6, *df*=4, *P*<0.001)		
Sham-operated *H. subflexa* male	22	0.84(0.104) A
Sham-operated *H. subflexa* male + hexane	14	0.75 (0.139) A
HP-ablated *H. subflexa* male	21	0.38 (0.112) BC
HP-ablated *H. subflexa* male + hexane	20	0.20 (0.132) C
HP-ablated *H. subflexa* male + 1ME *H. subflexa* HP extract	15	0.66 (0.133) AB
(b) Interspecific mating - *H. virescens* female with: (X^2=38.3, *df*=7, *P*<0.001)		
Sham-operated *H. subflexa* male	34	0.62 (0.085) BC
Sham-operated *H. subflexa* male + hexane	12	0.67 (0.076) BC
Sham-operated *H. subflexa* male + 1ME *H. virescens* HP extract	15	0.79 (0.077) B
HP-ablated *H. subflexa* male	17	0.29 (0.062) D
HP-ablated *H. subflexa* male + hexane	25	0.20 (0.054) D
HP-ablated *H. subflexa* male + 1ME *H. virescens* HP extract	18	0.50 (0.121) C
Control		
Control female *H. virescens* + male *H. virescens*	17	0.88 (0.080)AB
Control female *H. subflexa* + male *H. subflexa*	23	(0.000) A
(c) Interspecific mating - *H. subflexa* female with: (X^2=38.8, *df*=7, *P*<0.001)		
Sham-operated *H. virescens* male	22	0.18 (0.084) C
Sham-operated *H. virescens* male + hexane	11	0.30 (0.151) C
Sham-operated *H. virescens* male + 1ME *H. subflexa* HP extract	16	0.69 (0.123) B
HP-ablated *H. virescens* male	15	0.24 (0.092) C
HP-ablated *H. virescens* male + hexane	13	0.30 (0.080) C
HP-ablated *H. virescens* male + 1ME *H. subflexa* HP extract	24	0.67 (0.098) B
Control		
Control female *H. virescens* + male *H. virescens*	20	0.75 (0.115) B
Control female *H. subflexa* + male *H. subflexa*	20	0.95 (0.050) A

[a] Means with no letters in common are different (*P*<0.05) from others for mating frequency within each experiment

HP-ablated male *H. subflexa*–female *H. virescens* treatments (relative to treatments with HP-ablated males alone or HP-ablated males plus a hexane blank).

Frequency of abdominal extension and abdominal curling increased for treatments with sham-operated or HP-ablated *H. subflexa* males in the presence of *H. virescens* HP extract, relative to treatments without *H. virescens* HP extract (sham operated and HP-ablated males alone or with addition of hexane; Table 2), indicating that the presence of conspecific male HP extract caused increased occurrences of behaviors associated with successful mating. Frequency of clasping was increased in HP-ablated *H. subflexa* males in the presence of *H. virescens* HP extract relative to the addition of hexane. Pairings in the presence of *H. virescens* HP extract also showed a higher number of false clasps. Mean number of mating attempts by male *H. subflexa* was not different between treatments with or without *H. virescens* HP extract (Fig. 2a).

H. virescens Males and H. subflexa Females

Conspecific pairs of both species mated in high frequencies (Table 1). Mating frequencies between *H. virescens* males and *H. subflexa* females was low for sham-operated and HP-ablated males, with or without hexane. The presence of 1ME of *H. subflexa* HP extract with sham-operated or HP-ablated males resulted in increased mating acceptances, to levels similar to that of control *H. virescens* matings.

The presentation of *H. subflexa* HP extract along with a sham-operated *H. virescens* male resulted in changes in the frequencies of female behaviors linked with mate acceptance, compared to when *H. subflexa* HP extract was absent. This included reductions in flying and moving away, and increases in abdominal extension (Table 3). Abdominal curling increased in both HP-ablated and sham-operated treatments when *H. subflexa* HP extract was present (compared to when it was absent), and was not different

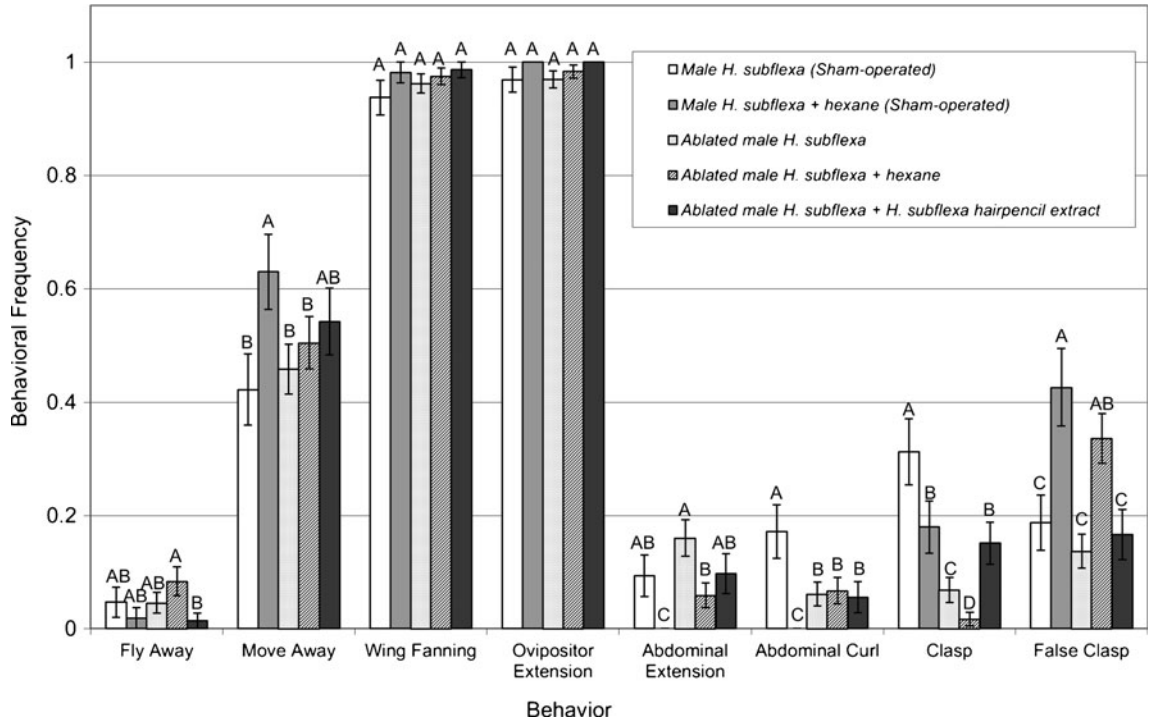


Fig. 1 Behavioral responses of female *Heliothis subflexa* to conspecific males with various treatments: sham-operated hair pencils (HPs), sham-operated HPs plus a hexane source, ablated HPs, ablated HPs with a hexane source, or ablated males with a one male equivalent of *H. subflexa* HP extract source. Means with the same letter are not different (at $P<0.05$). Behavioral differences among treatments: Fly away, $X^2=6.29$, $P=0.18$; Move away, $X^2=12.5$, $P<0.01$; Wing fanning, $X^2=3.36$, $P=0.50$; Ovipositor extension, $X^2=4.08$, $P=0.40$; Abdominal extension, $X^2=14.2$, $P<0.001$; Abdominal curl, $X^2=14.6$, $P<0.01$; Clasp, $X^2=41.2$, $P<0.001$; False Clasp, $X^2=27.0$, $P<0.001$ (4° of freedom in each case)

Table 2 Behavioral responses of female *Heliothis virescens* to male *H. subflexa* with various treatments involving sham operation or ablation of hair pencils (HPs), and additional sources of hexane or HP extract (one male equivalent = 1ME) of male *H. virescens*. Pairs of male and female *H. virescens*/*H. virescens* and *H. subflexa*/*H. subflexa* were used for comparison of mating frequencies. *Means with the same letter are not different (at $P<0.05$)

Treatment (degrees of freedom = 7)	Frequency of observed behaviors (± SEM)*						
	N	Fly away	Move away	Abdominal extension	Abdominal curl	Clasp	False clasp
		$X^2=6.88$, $P=0.23$	$X^2=12.8$, $P=0.02$	$X^2=12.0$, $P=0.03$	$X^2=9.83$, $P=0.08$	$X^2=38.3$, $P<0.001$	$X^2=14.4$, $P=0.01$
Sham-operated *H. subflexa* male	34	0.0 B	0.24 (0.066)BC	0.02 (0.02)B	0.05 (0.033)B	0.08 (0.046)C	0.21 (0.064)BC
Sham-operated *H. subflexa* male + hexane	12	0.0 B	0.22 (0.011)C	0.05 (0.05)B	0.O4 (0.036)B	0.11 (0.045)BC	0.32 (0.109)AB
Sham-operated *H. subflexa* male + 1ME *H. virescens* HP extract	15	0.03 (0.032)AB	0.26 (0.079)BC	0.16 (0.047)A	0.25 (0.072)A	0.15 (0.054)AB	0.41 (0.090)A
HP-ablated *H. subflexa* male	17	0.03 (0.032)AB	0.40 (0.021)B	0.047 (0.043)B	0.06 (0.041)B	0.06 (0.056)C	0.30 (0.110)AB
HP-ablated *H. subflexa* male + hexane	25	0.05 (0.051)AB	0.38 (0.060)B	0.05 (0.05)B	0.06 (0.036)B	0.07 (0.060)C	0.28 (0.108)AB
HP-ablated *H. subflexa* male + 1ME *H. virescens* HP extract	18	0.0 B	0.31 (0.061)B	0.16 (0.024)A	0.16 (0.045)AB	0.19 (0.050)AB	0.29 (0.060)AB
Control							
Control female *H. virescens* + male *H. virescens*	17	0.0 B	0.54 (0.075)A	0.04 (0.031)B	0.16 (0.056)AB	0.23 (0.059)A	0.34 (0.072)AB
Control female *H. subflexa* + male *H. subflexa*	23	0.05 (0.022)A	0.43 (0.053)AB	0.15 (0.038)A	0.09 (0.031)B	0.26 (0.047)A	0.13 (0.036)C

from the frequency observed in male and female *H. virescens* pairings. The mean number of mating attempts was greater for treatments with sham-operated and HP-ablated male *H. subflexa* compared to that in the control *H. subflexa*/*H. subflexa* pairing (Fig. 2b). Overall, the presence of *H. subflexa* HP extract resulted in reduced numbers of mating attempts by both HP-ablated and sham-operated males.

Discussion

Hairpencils and *H. subflexa*

Male HP volatiles are important in courtship and mate acceptance in *H. subflexa*; surgical removal of male *H. subflexa* HPs reduced the frequencies of clasping and corresponding mating acceptance relative to those of controls. In the presence of *H. subflexa* HP extract, the frequency of mating in operated males was restored to a level comparable to that of control pairs. Therefore, HP volatiles influence *H. subflexa* courtship and should not be considered purely vestigial. It is unclear what specific behavioral effect HP volatiles elicit in female *H. subflexa*, as there was no distinct qualitative difference in behavioral response when HP extract was present or absent. In *H. virescens*, HP volatiles caused a decrease in the frequency of moving away and an increase in abdominal extension and curling in females (Hillier and Vickers, 2004). In *H. subflexa*, abdominal extension and curling were rarely observed, even in control treatments, and HP volatiles did

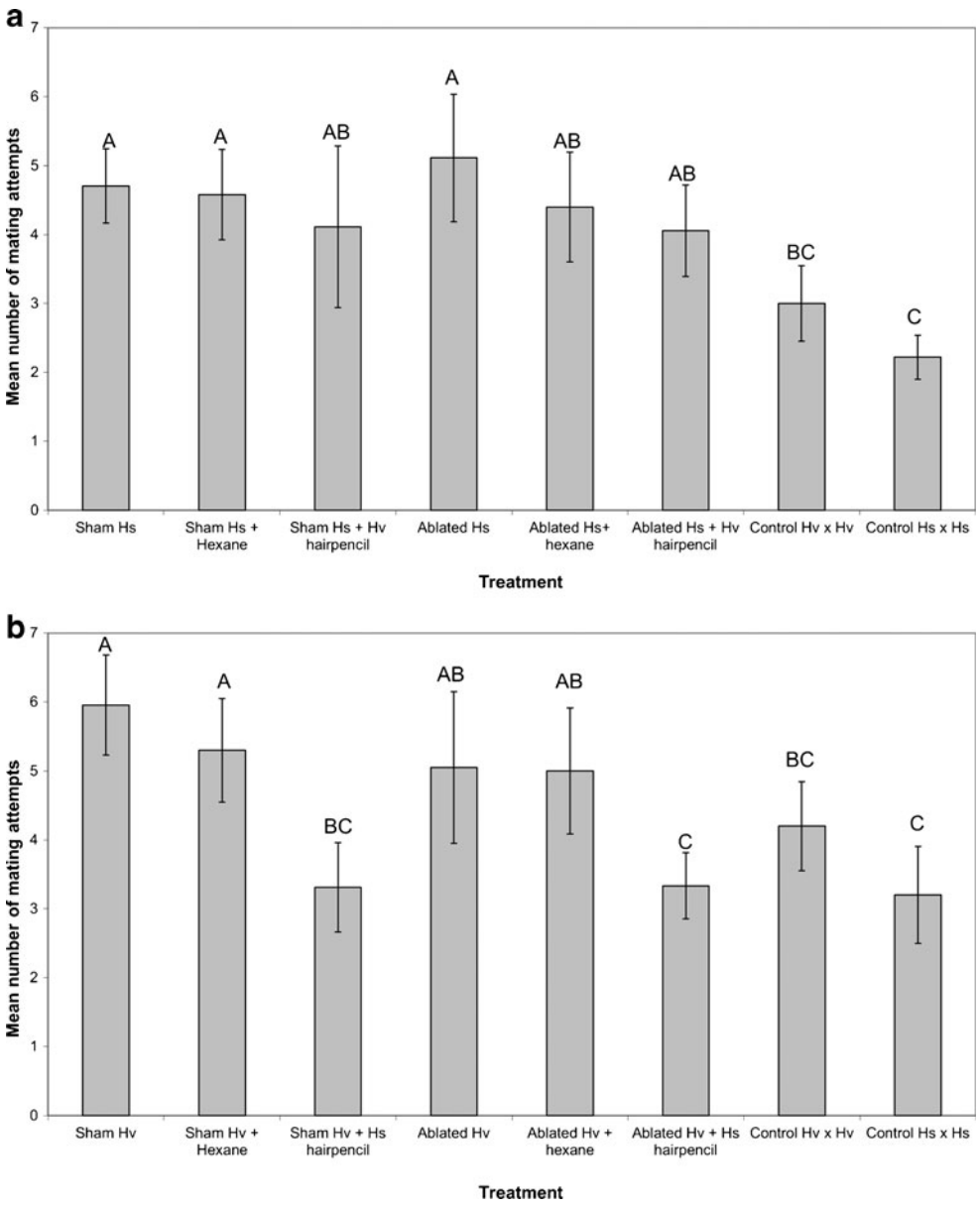


Fig. 2 **a** Mean number of mating attempts between female *Heliothis virescens* (Hv) and male *H. subflexa* (Hs) with various treatments: sham-operated hair pencils (HPs), sham-operated HPs plus a hexane source, sham-operated HPs plus one male equivalent of *H. virescens* HP extract, ablated HPs, ablated HPs plus a hexane source, ablated HPs plus one male equivalent of *H. virescens* HP extract. Pairs of male and female *H. virescens*/*H. virescens* and *H. subflexa*/*H. subflexa* were for comparison of mating frequency. **b** Mean number of mating attempts between female *H. subflexa* and male *H. virescens* with various treatments: sham-operated HPs, sham-operated HPs with a hexane source, sham-operated HPs plus a source of one male equivalent of *H. subflexa* HP extract, ablated HPs, ablated HPs plus a hexane source, ablated HPs plus one male equivalent of *H. subflexa* HP extract. Pairs of male and female *H. virescens*/*H. virescens* and *H. subflexa*/*H. subflexa* (N=20) were for comparison of mating frequency. For each experiment (**a** and **b**), means with the same letter are not different (at P<0.05)

not appear to inhibit females from moving away from courting males.

In spite of this, 38% of HP-ablated male *H. subflexa* mated successfully, indicating that while HP volatiles influence mating success, their absence does not preclude successful mating. In *H. virescens*, HP volatiles appear to have a greater influence on mating success, with <5% of females mating with HP-ablated males (Hillier and Vickers, 2004). The effect on mating by HP-ablated *H. subflexa* males is similar to that of other species, such as *E. elutella* and *Mamestra brassicae* L. (Lepidoptera: Noctuidae), in which HP volatiles improve mating success but are not essential for mating (Phelan et al., 1986; Birch et al., 1989). Possibly, other stimuli not recorded in this study, including visual and acoustic cues, might play a role in female mate acceptance in the absence of olfactory signals.

H. subflexa Males and *H. virescens* Females

The frequency of successful matings between *H. subflexa males* and *H. virescens females* was lower than that for the intraspecific mating of *H. subflexa*/*H. subflexa*. Since ablation of HPs of *H. subflexa* males further decreased the proportion of successful matings, and mating success of *H. subflexa* males courting *H. virescens* females increased in the presence of *H. virescens* HP extract, it suggests a role for these chemicals in prezygotic reproductive isolation between these species. The presence of conspecific HP volatiles during courtship caused an increase in the frequency of abdominal extension and curling by female *H. virescens*; both of these behaviors have been shown previously to correlate with mate acceptance in this species (Hillier and Vickers, 2004).

H. virescens Males and *H. subflexa* Females

Addition of *H. subflexa* HP extract caused an increase in the mating acceptance in both sham-operated and HP-ablated *H. virescens* males. HP extract caused reduced moving and flying away by females, and increased abdominal extension, compared to when extract was not present. The frequency of abdominal curling, a behavior rarely observed in *H. subflexa* mating, also increased in treatments in which HP extract was present, compared to those in which it was not present. The greater frequency of this behavior in the interspecific trials may be due to it being more difficult to observe with *H. subflexa* males because of their rapid, 'aggressive' courtship.

Hairpencils and Reproductive Isolation

Although these two species differ in (larval) host range, the overlap of the highly mobile adults likely increases the possibility of interspecific mating. Differences in the phenologies of adults also can help preclude interspecific matings, but both species are multi-generational, increasing

Table 3 Behavioral responses of female *Heliothis subflexa* to male *H. virescens* with various treatments involving sham operation or ablation of hair pencils (HPs), and additional sources of hexane or HP extract (one male equivalent = 1ME) of male *H. subflexa*. Pairs of male and female *H. virescens*/*H. virescens* and *H. subflexa*/*H. subflexa* were used for comparison of mating frequencies. *Means with the same letter are not different (at $P<0.05$)

Treatment (degrees of freedom = 7)	Frequency of observed behaviors (± SEM)*						
	N	Fly away	Move away	Abdominal extension	Abdominal curl	Clasp	False clasp
		$X^2=17.4$, $P<0.01$	$X^2=25.0$, $P<0.001$	$X^2=19.4$, $P<0.01$	$X^2=32.6$, $P<0.01$	$X^2=21.1$, $P<0.001$	$X^2=7.58$, $P=0.18$
Sham-operated *H. virescens* male	22	0.07 (0.022)A	0.53 (0.042)AB	0.01 (0.010)B	0.05 (0.018)B	0.04 (0.017)B	0.24 (0.036)BC
Sham-operated *H. virescens* male + hexane	11	0.06 (0.019)AB	0.49 (0.050)B	0.02 (0.024)B	0.05 (0.034)B	0.05 (0.044)B	0.23 (0.051)BC
Sham-operated *H. virescens* male + 1ME *H. subflexa* HP extract	16	0.0 C	0.25 (0.051)C	0.12 (0.030)A	0.22 (0.049)A	0.24 (0.050)A	0.34 (0.055)A
HP-ablated *H. virescens* male	17	0.03 (0.023)ABC	0.43 (0.073)B	0.02 (0.020)B	0.05 (0.028)B	0.05 (0.030)B	0.21 (0.034)C
HP-ablated *H. virescens* male + hexane	13	0.02 (0.020)ABC	0.46 (0.072)B	0.02 (0.020)B	0.04 (0.029)B	0.07 (0.028)B	0.20 (0.036)C
HP-ablated *H. virescens* male + 1ME *H. subflexa* HP extract	24	0.0 C	0.61 (0.065)A	0.03 (0.017)B	0.17 (0.052)A	0.16 (0.050)A	0.39 (0.066)A
Control							
Control female *H. virescens* + male *H. virescens*	20	0.02 (0.022)BC	0.60 (0.074)AB	0.0 C	0.24 (0.064)A	0.16 (0.054)A	0.36 (0.072)A
Control female *H. subflexa* + male *H. subflexa*	20	0.0 C	0.40 (0.050)B	0.09 (0.030)A	0.04 (0.020)B	0.18 (0.039)A	0.31 (0.047)AB

the likelihood of temporally overlapping cohorts (Stadelbacker and Martin, 1981). Thus, HP volatiles during courtship are likely a further barrier, in addition to differential attraction to the respective female-produced sex pheromones, to avoid interspecific mating. Redundancy in pre-mating species isolating mechanisms provides a means for reduced risk for mating mistakes in sympatry (Birch et al., 1990).

Recent studies have documented the inheritance, genetic architecture, neurophysiology, and behavioral phenotype of female sex pheromone preferences in male *H. virescens* and *H. subflexa*, along with associated hybrid and backcross progenies (Baker et al., 2006; Vickers, 2006a,b; Gould et al. 2010). These studies suggest strong linkages between specific genes coding putative olfactory receptor proteins, olfactory receptor neuron specificity, antennal lobe activity, and behavioral output of male moths relative to specific female sex pheromone components of each species. We previously identified an olfactory receptor neuron in *H. virescens* males and females that is sensitive to the male HP components hexadecyl acetate, octadecyl acetate, and to (*Z*)-11-hexadecenyl acetate (*Z*11-16:OAc; Hillier et al., 2006, 2007). In *H. virescens*, chemicals detected by this olfactory receptor neuron inhibit male upwind flight and thus function to repel competing males during courtship. The presence of *H. virescens* HP extract also causes a decrease in the overall number of mating attempts by *H. virescens* males (Hillier and Vickers, 2004). In this study, however, there were no differences in mating attempts by *H. subflexa* males, regardless of whether HP extract (of either species) was present or not (data not shown). This is curious, and is perhaps related to the presence of *Z*11-16:OAc in the *H. subflexa* female sex pheromone. Given the similarity in HP composition of the two species, future neurophysiological experiments should examine how such chemicals are detected by *H. subflexa*, to determine whether there are differences in how HP chemicals are processed between these closely related species.

Results of the current study may indicate a circumstance in which evolution of quiescent behavioral responses to both male and female sex pheromone components overlap in these species. Given their recent divergence, a mechanism of mate recognition and selection by females could be implicated in speciation between *H. virescens* and *H. subflexa*. Work by Lassance and Löfstedt (2009) suggests that analogies exist between male and female courtship signals in *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), wherein pleiotropy could influence coincident evolution of signals involved in mate selection. Other characters, including host plant choice, female sex pheromone composition, and male pheromone preference might have greater importance in such speciation, though selection for HP composition and female preference would be reinforced through assortative mating (Lassance and Löfstedt, 2009; Gould et al., 2010). Furthermore, the interaction of these traits may be supported by the fact that in many other insect species, male pheromone composition is dictated by sequestration of host plant volatiles (Birch et al., 1990).

In summary, this study provides evidence that HP volatiles are important in reproductive isolation and mate acceptance in *H. subflexa*. We previously demonstrated that *H. virescens* females discriminate between HP extracts of males of the two species (Hillier and Vickers, 2004). Our observations suggest that HP volatiles have similar effects on *H. subflexa* females as they do on *H. virescens* females: quiescence and arrestment (along with abdominal extension and curling).

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MS) using a Shimadzu GC17A − GCMS-QP5050A. The GC was equipped with a 30 m×0.25 mm CP-Sil 8CB fused silica capillary column (Varian Inc., Lake Forest, CA, USA). Helium was used as carrier gas, and the GC was programmed from 80°C for 5 min, then 8°C/min to 270°C. The injector temperature was set to 200°C. Mass spectra were acquired at 70 eV. Gas chromatographic analyses were carried out on a Shimadzu GC2014AFSC equipped with a flame ionization detector, and a 30 m× 0.25 mm RTX®-5 fused silica capillary column (Restek U.S., Bellefonte, PA, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX-400 spectrometer (400 MHz for ^{1}H, 100.6 MHz for ^{13}C) with TMS as internal standard.

Syntheses of Compounds All solvents and chemicals were purchased either from Sigma Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany). Silica gel for column chromatography was from Merck (silica gel 60, 63–200 μm; silica gel 60, 32–63 μm for flash chromatography). Dodecyl acetate (12:Ac) was obtained by acetylation (Ac_2O, DMAP, pyridine) of dodecan-1-ol, (*Z*)-5-tetradecenyl acetate (Z5-14:Ac), and (*E*)-5,13-tetradecadienyl acetate (E5,13-14:Ac) were prepared as described previously (Bergmann et al., 2007), and (*Z*)-5,13-tetradecadienyl acetate (Z5,13-14:Ac, **1**) was prepared as described below.

*8-(Tetrahydro-2H-pyran-2-yloxy)octan-1-ol (**2**)* PPTS (0.35 g, 1.4 mmol) was added to a solution of octane-1,8-diol (7.0 g, 47.9 mmol) and 4.55 ml 3,4-dihydro-2*H*-pyran (4.19 g, 49.8 mmol) in dry dichloromethane (30 ml), and the mixture was stirred at room temperature. After 5 hr of stirring, hexane (60 ml) and sat. $NaHCO_3$ solution (20 ml) were added to the mixture. After 10 min stirring, the phases were separated, and the aqueous phase was extracted with Et_2O. The combined organic phases were dried (Na_2SO_4), and the solvent was removed under vacuum. The residue was purified by chromatography on silica gel (hexane-ethyl acetate 1:1), yielding 7.25 g (65%) of **2** as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 4.57 (t, 1H, J=3.5 Hz), 3.91-3.83 (m, 1H), 3.72 (dt, 1H, J=9.7, 6.9 Hz), 3.63 (t, 2H, J=6.6 Hz), 3.53-3.46 (m, 1H), 3.38 (dt, 1H, J=9.7, 6.9 Hz), 1.87-1.77 (m, 1H), 1.75-1.67 (m, 1H), 1.65-1.48 (m, 8H), 1.40-1.27 (m, 8H). ^{13}C NMR ($CDCl_3$): δ 98.9, 67.7, 63.1, 62.4, 32.8, 30.8, 29.7, 29.4, 29.3, 26.2, 25.7, 25.5, 19.7. MS (EI, 70 eV): *m/z* 39 (37), 41 (88), 42 (26), 43 (31), 53 (12), 54 (28), 55 (100), 56 (37), 57 (28), 67 (29), 68 (14), 69 (26), 85 (37), 101 (5), 230 (M^+, <1).

*8-(Tetrahydro-2H-pyran-2-yloxy)octanal (**3**)* To a stirred solution of 8.30 ml oxalyl chloride (12.5 g, 98.0 mmol) in dry dichloromethane (50 ml) at −78°C, 18.0 ml DMSO (19.8 g, 254 mmol) in dichloromethane (50 ml) were slowly added under nitrogen atmosphere. After 30 min of stirring, a solution of **2** (7.20 g, 31.3 mmol) in dichloromethane (20 ml) was added dropwise. After further 40 min stirring, 52.0 ml Et_3N (38.0 g, 376 mmol) were added, the mixture was left stirring at room temperature for 1 hr, treated with H_2O, and extracted with Et_2O. The organic phase was dried (Na_2SO_4), and the solvent was removed under vacuum. The residue was purified by chromatography on silica gel (hexane-ethyl acetate 5:1), giving compound **3** as a colorless oil (6.67 g, 93%). ^{1}H NMR ($CDCl_3$): δ 9.74 (t, 1H, J=2.0 Hz), 4.56-4.52 (m, 1H), 3.88-3.80 (m, 1H), 3.70 (dt, 1H, J=9.3, 6.8 Hz), 3.51-3.44 (m, 1H), 3.35 (dt, 1H, J=9.3, 6.3 Hz), 2.40 (dt, 2H, J=7.3, 1.5 Hz), 1.84-1.75 (m, 1H), 1.72-1.65 (m, 1H), 1.65-1.46 (m, 8H), 1.40-1.28 (m, 10H). ^{13}C NMR ($CDCl_3$): δ 202.8, 98.8, 67.5, 62.3, 43.8, 30.7, 29.6, 29.1, 29.0, 26.0, 25.4, 21.9, 19.6. MS (EI, 70 eV): *m/z* 39 (12), 41 (69), 42 (11), 43 (29), 55(47), 56 (36), 57 (28), 67 (43), 84 (14), 85 (100), 98 (3), 101 (24), 109 (17), 127 (1), 228 (M^+, <1).

*Tetrahydro-2-(non-8-enyloxy)-2H-pyran (**4**)* Under an inert atmosphere, a solution of $NaN[Si(Me)_3]_2$ in THF (1 M, 30.0 ml, 30.0 mmol) was added to a suspension of methyl triphenyl phosphonium bromide (10.6 g, 29.7 mmol) in dry THF (100 ml). The mixture was stirred at −20°C for 30 min. The solution then was cooled to −78°C, and **3** (6.65 g, 29.3 mmol) was slowly added. The mixture was allowed to warm to room temperature, stirred for 30 min, then poured into ice-water and extracted with Et_2O. The organic phase was washed with brine, dried (Na_2SO_4), evaporated to near dryness, and chilled to precipitate most of the Ph_3PO. After filtration and washing of the precipitate with hexane, the filtrate was concentrated under vacuum. The residue was purified by chromatography on silica gel (hexane- ethyl acetate 5:1) to yield 4.01 g (61%) of **4** as colorless oil. ^{1}H NMR ($CDCl_3$): δ 5.82 (ddt, 1H, J=17.1, 10.3, 6.9 Hz), 4.98 (d, 1H, J=17.0 Hz), 4.92 (d, 1H, J= 10.3 Hz), 4.57 (m, 1H), 3.91-3.84 (m, 1H), 3.73 (dt, 1H, J= 9.6, 6.6 Hz), 3.54-3.47 (m, 1H), 3.39 (dt, 1H, J=9.6, 6.6 Hz), 2.08-2.00 (m, 2H), 1.88-1.80 (m, 1H), 1.76-1.70 (m, 1H), 1.69-1.47 (m, 6H), 1.42-1.28 (m, 8H). ^{13}C NMR ($CDCl_3$): δ 139.2, 114.1, 98.9, 67.7, 62.4, 33.8, 30.7, 29.7, 29.3, 29.0, 28.8, 26.1, 25.5, 19.6. MS (EI, 70 eV): *m/z* 39 (11), 41 (57), 43 (19), 55 (33), 56 (27), 57 (14), 67 (18), 69 (27), 83 (15), 85 (100), 101 (17), 226 (M^+, <1).

*Non-8-en-1-ol (**5**)* Compound **4** (4.00 g, 17.7 mmol) was added to a solution of *p*-toluenesulfonic acid in MeOH (50 ml), and the reaction mixture was stirred at room temperature for 4 hr. The solvent was evaporated, and the resulting residue was dissolved in CH_2Cl_2 (25 ml). The organic phase was washed with brine, dried (Na_2SO_4), concentrated, and the residue was chromatographed on

silica gel (hexane:ethyl acetate 4:1) to yield **5** (1.69 g, 67%). ^{1}H-NMR ($CDCl_3$): δ 5.81 (ddt, 1H, J=17.0, 10.3, 7.2 Hz), 4.99 (dd, 1H, J=17.0, 1.9 Hz), 4.93 (dd, 1H, J=10.3, 1.9 Hz), 3.64 (t, 2H, J=6.6 Hz), 2.04 (overlapped dt, 2H, J~7.4 Hz), 1.62-1.52 (m, 2H), 1.42-1.28 (m, 9H). ^{13}C NMR ($CDCl_3$): δ 139.1, 114.2, 63.1, 33.8, 32.8, 29.3, 29.1, 28.8, 25.7. MS (EI, 70 eV): *m/z* 39 (35), 41 (100), 42 (19), 43 (20), 54 (49), 55 (77), 57 (13), 67 (46), 68 (32), 81 (29), 82 (20), 95 (11), 96 (5), 109 (1), 124 (1), 142 (M^+, <1).

*Non-8-enal (**6**)* In a procedure essentially similar to the synthesis of **3**, 3.58 ml (5.37 g, 41.0 mmol) oxalyl chloride, 6.3 ml DMSO (6.93 g, 89.5 mmol), 5.74 g of **5** (37.3 mmol), and 26.0 ml (18.87 g, 186.5 mmol) Et_3N were reacted to give 4.25 g **6** as slightly yellow oil (75%) The crude product was used in the next step without purification. ^{1}H NMR ($CDCl_3$): δ 9.77 (t, 1H, J=2.0 Hz), 5.80 (ddt, 1H, J=17.0, 10.3, 7.0 Hz), 5.00 (dd, 1H, J=17.0, 1.8 Hz), 4.94 (dd, 1H, J=10.3, 1.8 Hz), 2.42 (dt, 2H, J=7.3, 2.0 Hz), 2.04 (overlapped dt, 2H, J~7.3 Hz), 1.68-1.59 (m, 2H), 1.42-1.28 (m, 6H). ^{13}C NMR ($CDCl_3$): δ 202.9, 138.9, 114.3, 43.9, 40.8, 33.6, 29.0, 28.8, 22.0. MS (EI, 70 eV): *m/z* 41 (85), 43 (33), 54 (54), 55 (100), 56 (20), 57 (23), 67 (25), 68 (22), 69 (11), 70 (12), 79 (15), 80 (14), 81 (24), 93 (10), 98 (12), 111 (3), 140 (M^+, <1).

*5-Acetoxypentyl Triphenyl Phosphonium Bromide (**8**)* A solution of 5-bromopentyl acetate (4.0 g, 19.2 mmol) and triphenylphosphine (5.0 g, 19.2 mmol) in dry acetonitrile (30 ml) was stirred under reflux for 24 hr. After cooling to room temperature, the solvent was evaporated under vacuum. Flash chromatography of the resulting residue on a silica gel column using (CH_2Cl_2 — MeOH 8:1) yielded 7.5 g (84%) of **8** as a viscous yellow oil. To assure solubility of the sample, the NMR spectrum was acquired in methanol-D4. The signals appeared broadened and are reported as multiplets, as coupling constants could not be determined. ^{1}H NMR (CD_3OD): δ 7.95-7.62 (m, 15H), 4.08-4.00 (m, 2H), 3.50-3.36 (m, 2H), 2.00 (s, 3H), 1.78-1.56 (m, 6H).

*(Z)-Tetradeca-5,13-dienyl Acetate (**1**)* A solution of NaN[$Si(Me)_3$]$_2$ in THF (1 M, 2.6 ml, 2.6 mmol) was added to **8** (1.20 g, 2.55 mmol) in dry THF (50 ml) at −20°C in N_2 atmosphere. The mixture was stirred for 30 min. The solution then was cooled to −78°C, and **6** (0.35 g, 2.55 mmol) was added dropwise. The reaction mixture was stirred for additional 2 hr at −78°C, and 1 hr at room temperature. The reaction mixture was poured into ice-water and extracted with Et_2O. The organic phase was washed with brine, dried (Na_2SO_4), and evaporated to dryness to yield a residue that was purified by chromatography on silica gel (hexane-ethyl acetate 20:1), giving **1** as colorless oil (227 mg, 36%). Chemical purity (GC) >98%, isomeric purity (GC): 94% Z, 6% E. Traces of the geometric isomer were removed by argentation chromatography as follows. To a solution of 5 g $AgNO_3$ in 15 ml CH_3CN, 15 g silica gel were added, the mixture was ground for 5 min, and the beaker was covered completely with aluminum foil, and held at 70°C overnight resulting in the evaporation of the solvent. The slightly grayish silica was used for chromatography as usual, covering the column with aluminum foil (Li et al., 1995). ^{1}H NMR ($CDCl_3$): δ 5.81 (ddt, 1H, J=17.0, 10.4, 7.4 Hz), 5.42-5.29 (m, 2H), 4.99 (dd, 1H, J=17.0, 1.9 Hz), 4.93 (dd, 1H, J=10.4, 1.9 Hz), 4.06 (t, 2H, J=7.0 Hz), 2.09-1.98 (m, 6H), 2.05 (s, 3H), 1.68-1.59 (m, 2H), 1.44-1.24 (m, 10 H). ^{13}C NMR ($CDCl_3$): δ 171.2, 139.2, 130.5, 129.1, 114.1, 64.5, 33.8, 29.6, 29.1, 29.0, 28.9, 28.2, 27.2, 26.7, 26.0, 20.9. The NMR data were in general agreement to those reported earlier in CCl_4 as solvent (Cahiez et al., 1980). MS (EI, 70 eV): *m/z* 41 (67), 43 (100), 54 (56), 55 (62), 67 (68), 68 (31), 79 (26), 80 (32), 81 (44), 82 (23), 93 (14), 94 (15), 95 (28), 96 (13), 107 (8), 110 (8), 121 (11), 135 (7), 149 (3), 163 (1), 192 (1), 252 (M^+, <1).

Laboratory Bioassays Larvae of the last instar (Olivares and Angulo, 1992) were removed from the plastic cups, and held individually under bioassay conditions (20°C) 1 hr prior to assay. Behavioral assays used a glass Y-tube olfactometer (3.5 cm i.d., 20 cm arms) coupled via Teflon tubing (4 mm i.d., 70 cm in length) to two 1 l glass containers. The Y-tube was placed horizontally, and the inside bottom was provided with screen in appropriate dimensions to facilitate movement of larvae. Charcoal-filtered air was supplied at a rate of 1.5 $lmin^{-1}$, prior optimization using NH_3/HCl to visualize the air flow (Baker and Linn, 1984). One of the glass containers contained the stimulus and the other either air only (first experiment) or solvent-treated filter paper (second and third experiment) as control. A single larva was placed at the base of the main olfactometer tube, its behavior was observed for 7 min and its choice recorded. Larvae that did not make a choice were excluded from statistical analysis. The stimulus source was rotated randomly to avoid positional bias. The olfactory responses of larvae to the following stimuli were assayed:

1. *Live conspecific larvae* Either 10 or 1 larvae of the last stage were separated and placed into the glass odor container 60 min before starting the assays.
2. *Hexane extract of larvae* A 5×5 cm piece of filter paper was loaded with either 510, 51, or 5.1 mg extract, corresponding to 10, 1, or 0.1 larval extract equivalent (LEE), respectively, 1 LEE being the amount of extract obtained when extracting 1 larva in hexane. After evaporation of the solvent, the paper was placed in the

glass container. Each treated filter paper was used in 5 experiments.

3. *Synthetic compounds* Synthetic compounds were assayed individually in amounts equivalent to their proportion in 1 LEE. Filter paper pieces were loaded with 22 mg Z5,13-14:Ac, 19 mg of 12:Ac, and 7 mg of Z5-14:Ac, respectively.

Data were analyzed by *Chi-square* test using Statistica 7.0 software.

Results

Synthesis of (Z)-5,13-tetradecadienyl acetate The individual reactions used to obtain **1** are summarized in Fig. 1. Swern oxidation of monoTHP-protected (Nishiguchi et al., 2000) octane-1,8-diol (**2**) yielded aldehyde **3,** which was chain-elongated by Wittig reaction using methyl triphenylphosphonium bromide (28% over three steps). Deprotection (67%) of resulting THP-protected 8-nonen-1-ol (**4**) followed by another Swern oxidation gave access to 8-nonenal (**6**), which was used in the next step without further purification. In parallel, 5-bromopentyl acetate was transformed into Wittig salt **8** by treatment with triphenylphosphine in MeCN (84%). Wittig reaction between **6** and **8** under salt-free conditions (Bestmann et al., 1976) yielded target compound **1** with about 6% of the geometrical isomer which was effectively removed by argentation chromatography.

Identification of Compounds Z5,13-14:Ac and E5,13-14:Ac were separated by GC, with the *E* isomer eluting later on a non-polar column (Retention indices 1786 and 1790, respectively, on RTX-5). The retention time of the main compound in hexane extracts of larvae matched that of the *Z* isomer (Fig. 2). Matching of mass spectra for the natural and synthetic materials was confirmed earlier (Bergmann et al., 2007).

Laboratory Bioassays The olfactory responses of larvae to different stimuli in a Y-tube olfactometer are summarized in Table 1. Larvae were attracted to 10 (χ^2=6.545, *df*=1, *P*=0.011) or 1 (χ^2=5.000, *df*=1, *P*=0.025) conspecifics. Hexane extracts of larvae in doses of 10 LEE (χ^2=10.714, *df*=1, *P*=0.001) and 1 LEE (χ^2=7.200, *df*=1, *P*=0.007) were attractive as well, but a dose of 0.1 LEE (χ^2=0.200, *df*=1, *P*>0.05) showed no attractiveness. Of the three major compounds identified in the hexane extract and tested at a 1 LEE dose, only Z5,13-14:Ac was attractive (χ^2=8.048, *df*=1, *P*=0.005), while 12:Ac (χ^2=0.297, *df*=1, *P*>0.05) and Z5-14:Ac (χ^2=0.200, *df*=1, *P*>0.05) were not attractive.

Discussion

Last-instars of *Ch. valdiviana* produce and are attracted to (*Z*)-5,13-tetradecadienyl acetate. The identification of the compounds present in hexane extracts of larvae of *Ch. valdiviana* has been described earlier (Bergmann et al., 2007); however, the identification of the main compound Z5,13-14:Ac was based on the mass spectrum and retention time of minor amounts of this compound in a sample of the geometric isomer E5,13-14:Ac. With the pure synthetic material in hand, we now confirm the identification.

Similar compounds have been found in other cossid species. Although the exact composition of the secretions vary, Z5,13-14:Ac, together with other compounds, also is produced by larvae of *Cossus cossus* (Trave et al., 1966), *Zeuzera pyrina* (Marchesini et al., 1969), and *Chilecomadia moorei* (Garbarino and Gambado, 1988). Apart from the chemical composition of these secretions, the respective

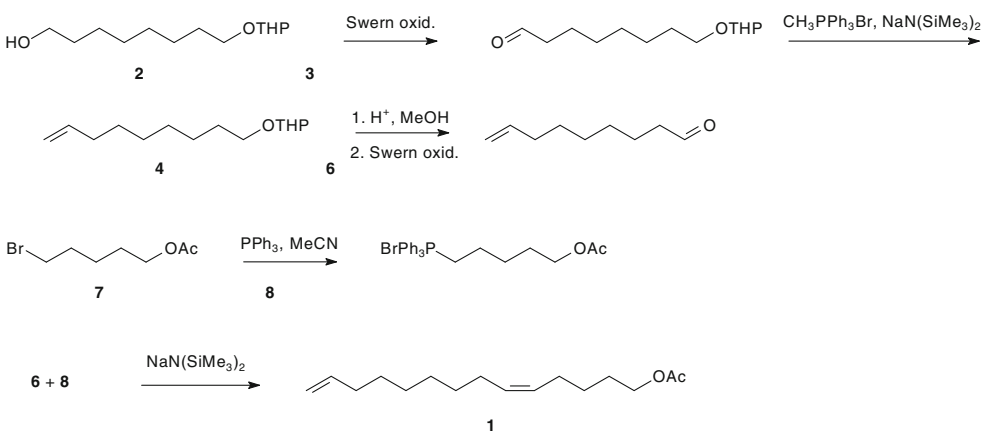


Fig. 1 Synthesis of (*Z*)-5,13-tetradecadienyl acetate

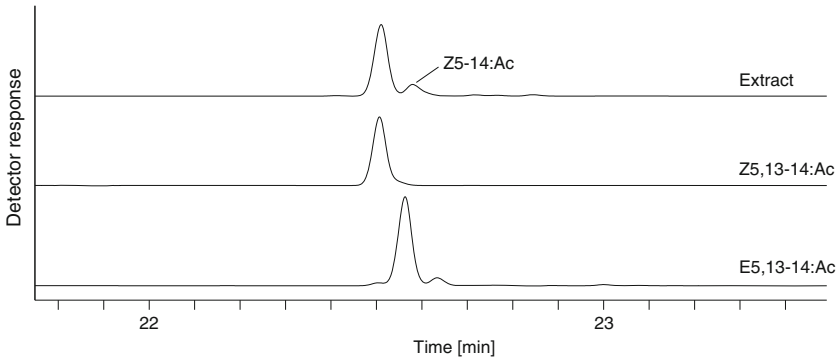


Fig. 2 Gas chromatograms of a hexane extract of *Chilecomadia valdiviana* larvae (*top*), synthetic samples of (*Z*)- (*middle*), and (*E*)-5,13-tetradecadienyl acetate (*bottom*)

reports contain no information about function. To give conclusive answers as to the role of the larval secretion in *Ch. valdiviana*, knowledge of the biology of the insect is needed, but little information is available in the literature. One report presents preliminary conclusions drawn from observations made during the 1992/1993 season (Cerda and Lewis, 1994) indicating, for example, that the life cycle is possibly completed in 2 or 3 years, with adults emerging from November — January. The same report highlights the gregarious habit of larvae, which translates into a magnification of the damage in host trees, due to the concentration of galleries in the affected zone. The present study links this behavior to the chemicals previously identified.

The adaptive role of this communication under natural conditions, however, still needs to be clarified. Taking into consideration available reports, it seems possible that mass attack may facilitate overcoming the defenses of a host tree. During the study, we observed that larvae have a strong tendency to wander when not inside galleries or covered by sawdust, which would facilitate aggregation (and also explain the high number of responders in the laboratory bioassays). The possibility of higher reproductive success when larvae pupate in aggregations, as suggested for *Cydia pomonella* (Duthie et al., 2003), cannot be excluded, but seems less likely as there is no evidence in *Ch. valdiviana* of inter-stage communication (e.g., larva-adult or pupa-adult). In particular, no adult moths were caught in traps baited with varying doses of larval extract during a preliminary field trial (unpublished results). It is also noteworthy that the sex pheromones produced by adult females of *C. cossus* and *Z. pyrina* consist of compounds different from those found in the respective larvae (Capizzi et al., 1983; Castellari, 1986).

This is the second example of a larval aggregation pheromone in Lepidoptera. A volatile aggregation pheromone was identified previously in *Cydia pomonella*, where a blend of compounds produced by cocoon-spinning larva containing (*E*)-2-octenal, (*E*)-2-nonenal, sulcatone, geranylacetone, 3-carene, octanal, nonanal, and decanal, attracts conspecific larvae (Jumean et al., 2005a), while a similar blend (heptanal, octanal, nonanal, decanal, (*E*)-2-octenal, 3-carene, sulcatone, and geranylacetone) was attractive to a larval parasitoid (Jumean et al., 2005b). Another example of chemical communication in lepidopteran larvae is the pheromone-mediated trail following and group foraging behavior exhibited by some social caterpillars (see, for example, Fitzgerald, 2003). In some species, the compound eliciting this behavior has been identified as 5β-cholestan-3-one (Fitzgerald, 1993; Ruf et al., 2001).

Synthesis of Z5,13-14:Ac has been described before, for example by taking advantage of the stereospecific addition of lithium alkyl cuprates to acetylene, followed by alkylation of the *Z*-configured vinylic cuprate (Cahiez et al., 1980). Our approach uses well-known reactions and avoids the use of acetylene and the carcinogenic solvent HMPT in the alkylation step, by employing Wittig reactions.

In summary, we showed that (*Z*)-5,13-tetradecadienyl acetate is the compound responsible for the aggregation of the larval stage of *Chilecomadia valdiviana*.

Table 1 Behavioral response of larvae of *Chilecomadia valdiviana* to conspecific larvae, hexane extract of conspecific larvae, and single synthetic compounds in a Y-tube olfactometer

	Stimulus	N	No. of larvae responding to	
			Stimulus[a]	Control
Exp. 1	10 larvae	25	17*	5
	1 larva	22	15*	5
Exp. 2	Extract (10 LEE)	25	18*	3
	Extract (1 LEE)	24	16*	4
	Extract (0.1 LEE)	25	9	11
Exp. 3	Z5,13-14:Ac (1 LEE)	24	17*	4
	12:Ac (1 LEE)	31	9	14
	Z5-14:Ac (1 LEE)	20	9	11

[a] Statistically significant differences in responses ($P<0.05$ according to *chi-square* test) are marked with *

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additional compounds that give prolonged activity. However, no additional active compounds have been identified.

Z9-16:Ald is known also as a component of some moth sex pheromones (Nesbitt et al., 1975; El-Sayed, 2011), and is commercially available. The use of trail pheromone to reduce or eliminate foraging, based on the moth mating disruption paradigm (Bartell, 1982; Witzgall et al., 2010), has been examined for control of Argentine ants (Tatsuki et al., 2005; Suckling et al., 2008; Tanaka et al., 2009; Nishisue et al., 2010; Sunamura et al., 2011). According to studies that use a single point source, and that are confirmed with a dispersed formulation in small plots outdoors, trail disruption acts by changing the walking track angle of ants within a treated area (Suckling et al., 2008). At close range to a point source, disruption results in a significant drop in trail integrity of individual ants, defined by the linear regression statistic, r^2, which is very high (0.9–1.0) on straight trails. High amounts of pheromone presented near a trail led to a build up of ants in the area; as ants entered the disrupted area, they lost the trail they were following and had difficulty leaving the area because they could not find the other end of the trail back to the food or nest. When pheromone was applied over a wide area, using dispensers or widely dispersed micro-encapsulated sources, ant foraging was reduced (Suckling et al., 2010; Sunamura et al., 2011). To be a successful control tactic, trail pheromone disruption must reduce foraging efficiency and suppress ant populations.

Several substrates for controlled release of trail pheromone have been tested for Argentine ants. Tanaka et al. (2009) used polyethylene tubing dispensers and, like Nishisue et al. (2010), found that small plots were reinvaded readily when the dispensers were removed. Disruption of trails lasted as long as pheromone was released in the small plots, but the practicality of hand applying polyethylene tubing dispensers 40 cm above ground may be limited. Suckling et al. (2008) used a short-lived formulation of sand plus trail pheromone in 1 and 4 m^2 plots to demonstrate another control approach: disruption of the boundary layer where ants are active. Suckling et al. (2010) used micro-encapsulated, widely dispersed, point sources and extended the longevity of the disruptant effect to 2 weeks. These methods could offer different approaches for intervention against invading ants, (Sunamura et al., 2011). However, more information is needed on the mechanisms, concentrations, and active components required for success of trail pheromone disruption for ant control.

Ant trail pheromone concentration likely is to be linked to the density of ants trailing, and the quality or enduring nature of the food resource (Deneubourg et al., 1990; Jackson and Châline, 2007), as successful foraging individuals reinforce the trail back to the nest (Van Vorhis Key and Baker, 1986). Here, we hypothesized that trail pheromone disruption should be related to initial trail pheromone concentration, in the same way that moth pheromone disruption is more successful at lower insect density (Witzgall et al., 2010). We also were interested in observing switching of trails to higher concentration ones, and the capacity for recovery at specific concentrations, because this might provide insight into the phenomenon, by indicating any effect of adaptation or sensory overload, a mechanism relevant to moth disruption, especially with off-ratio blends (Minks and Cardé, 1988).

Methods and Materials

Insects Five queen-right Argentine ant colonies were used and maintained by methods adapted from that described for the red imported fire ant, *Solenopsis invicta* (Banks et al., 1981). Colonies were field collected from Hoon Hay, Christchurch 43°34S 172°35E in December 2009, April and November 2010. Colonies were starved of protein and carbohydrates 2–3 d prior to the start of experiments, but allowed access to water, to encourage foraging.

Chemicals Z9-16:Ald (94.5% chemical purity) was purchased from Bedoukian Research (Danbury, CT, USA), and diluted in ethanol for use.

Experimental Set-up We hypothesized that concentrations required for disruption should depend on the strength of the base foraging trail (Trail 1), and that disruption of foraging by a second upwind stimulus trail (Trail 2) at the same concentration, should occur independent of trail strength. We used the approach reported by Suckling et al. (2008), based on the parallel trails used by Morgan et al. (2006), of a suspended horizontal glass plate (400×200×5 mm) as the substrate. This was connected by a single wire at one diagonal end to channel and regulate the foraging of an ant between nest and food source. The rectangular plate was positioned so as to allow airflow from Trail 2 across Trail 1 (Supplemental Fig. A, Fig. 1). A white background, with faint pencil lines drawn on paper under the glass, was used to maximize contrast. Trails were filmed diagonally overhead (i.e., at 45°) with a webcam (Logitech Pro 9000 webcam, Freemont, CA, USA; screen size 80% of actual, 960×720 pixels at 15 frames per second). This allowed viewing of the maximum length of the trail on the glass and the determination of trail integrity (defined as a significant value of r^2) of the base trail. The base trail (Trial 1, 30 cm length) of Z9-16:Ald was applied at two rates (1 and 100 pg/cm), by streaking 3 μl of pheromone in ethanol, from a glass capillary tube, in a straight line. Following application of the base trail, Trail 2 (10 cm length) was

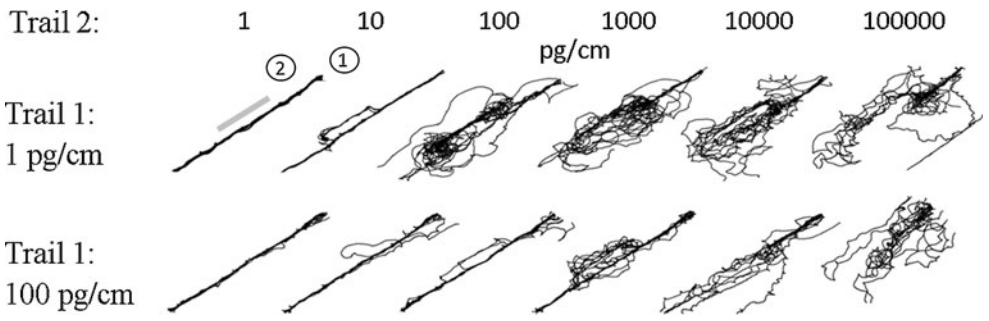


Fig. 1 Combined tracks of Argentine ants (N=10), *Linepithema humile*, at low and high (1 or 100 pg/cm) base-trail (Trail 1, 30 cm long) pheromone concentrations, disrupted by an upwind trail (Trail 2, shown as grey, wide line in top left image) of various concentrations (1–100,000 pg/cm) of (*Z*)-9-hexadecenal

drawn parallel, 1.5 cm upwind of the base trail, at six rates (1, 10, 100, 1,000, 10,000, and 100,000 pg/cm). Low speed directional air movement (~0.05 m.sec^{-1}) was generated by conducting the experiment 1.5 m from a fume hood. This airflow occurred at a ~60° angle to increase the angle of incidence (from 90° to >120°) for ants affected by Trail 2 (from the top down in Supplemental Fig. A). Experiments were conducted for a maximum of 30 min., or until 10 individuals were recorded on an outward journey from one colony. This was repeated five times for two base-trail concentrations, at six disruption rates, using 5 different colonies (N=50 ants per treatment). A dental roll with 10% sugar water was placed at the end of the trail downwind to provide a reward and to assess the success of forager ants in discovering this food source, as well as reducing the incidence of ants walking back onto the glass sheet. Only ants leaving the nest were recorded. This was done to reduce the influence of any additional cues to the trail pheromone that returning ants may have used to return to the nest (e.g., Cavill et al., 1979).

Walking of an individual ant was analyzed with MaxTraq v1.9 (Innovision Systems, Lapeer, MI, USA), using a minimum of 30 and a maximum of 600 recorded frames. A general linear model was conducted on the effect of base trail (Trail 1), stimulus trail (Trail 2), and their interaction, on disruption, measured as residual error sums of squares obtained by using the least squares regression method. Stimulus trail (Trail 2) concentrations of 100, 1,000, 10,000, and 100,000 pg/cm were analyzed, with the lower concentrations excluded, as there was no effect observed. The residual error sums of squares values were used instead of the r^2 trail integrity statistic (bounded by 0 and 1), due to the unsuitability of r^2 values to meet the assumptions of normal distribution and homogeneity of variance of the general linear model. The residual error sums of squares values are, in effect, a distance measure in one dimension, as they are calculated from the vertical distance from the line to the point and not the perpendicular distance. A square root transformation of the residual error sums of squares was used to meet the assumptions of normal distribution and homogeneity of variance of the general linear model. A second analysis was conducted, with colony added as a repeated factor, to examine any effect on disruption measures. Ant arrival success was recorded in binary form (success or failure), and a binary logistic regression was conducted with ant arrival success rate as the response, and base trail (Trail 1), stimulus trail (Trail 2), and their interaction as factors. The low concentration tests of Trail 2 were discarded, as for the general linear model. The percentages of ants that stayed on the base trail (Trail 1), switched to the disrupting trail (Trail 2), or were fully disrupted (never followed any trail), were recorded and compared.

Results

Preliminary tests indicated that the trail-inducing range of Z9-16:Ald on glass was substantially higher than that reported by Van Vorhis Key and Baker (1982b) using paper. They reported activity above the solvent control at approximately 0.2 pg/cm, and optimal trail following (mimicking the natural product) between 2,000 and 20,000 pg/cm, using a trail pheromone purity of >98%. Our results, however, indicated optimal trail-following integrity between 1 and 100 pg/cm and a loss in trail-following integrity outside of this range.

Individual ants tracked the 1 pg/cm and 100 pg/cm base trails consistently (Fig. 1). When the second trail was added upwind, ants showed a range of tracks, depending on the concentration of both the base (Trail 1) and stimulus (Trail 2) trails. When Trail 2 was 100-fold higher in concentration than Trail 1, tracks had erratic direction and changes in track angle (Fig. 1). The general linear model on residual error sums of squares showed that Trail 1 and Trail 2 both had an effect on trail integrity (Trail 1, $F_{(1,3,3)}$=55.88, P<0.001, Trail 2$F_{(1,3,3)}$=30.89, P<0.001), with no interaction between Trails 1 and 2 ($F_{(1,3,3)}$=0.44, P=0.726).

Adding colony as a repeated factor made little difference to the result ($F_{(4)}$=1.83, P=0.151). A similar result was obtained from the binary logistic regression conducted on ant success data, with Trail 1 and Trail 2 both having an effect on bait discovery success (Trail 1, P<0.001, Trail 2, P=0.002); there was no interaction (P=0.091) between the two trail effects.

At 1 pg/cm base trail, there was no evidence of ants switching to the parallel trail at the same concentration. Switching did not occur until the disruption trail (Trail 2) was 10–100 times greater in concentration than the base trail. At 100 pg/cm base trail, switching occurred when the disruption trails was also 100 pg/cm, and occurred more frequently when the disruption trail was 10–100 times more concentrated. Of the relatively few ants that switched between trails, when Trail 2 was not inducing full disruption, several returned successfully to the base trail before they reached the end of the disruption trail (Fig. 1).

Plotting the trail integrity statistic (r^2) against Trail 2 concentration (Fig. 2), showed the concentration difference between the two trails that was required for disruption of trail following on the base trail. At 1 pg/cm, no change in trail integrity on the base trail occurred at the two lowest concentrations of the disruption trail. However, once the disruption trail concentration reached 100 pg/cm, trail integrity on the base trail decreased. Similarly, at 100 pg/cm base-trail concentration, disruption was evident when the disruption trail was 10,000 pg/cm. Therefore, disruption of trail following on the base trail required a 100-fold greater concentration of the disruption trail.

Similar results were obtained for the arrival success of workers at the other end of a trail, after passing through the treated area (Fig. 2); i.e., a reduction in arrival was apparent only when the disruption trail concentration was roughly 100 times that of the base trail. There was a logarithmic relationship ($y=1.1076e^{4.513x}$ r^2=0.97) between arrival success and trail integrity (r^2), and general agreement in results for the two base-trail concentrations (Fig. 3). Changes in arrival success were more greatly affected by disruption than changes in trail integrity (e.g., when r^2 was ~0.6 the arrival success of workers at food sources was only ~15%).

Disruption and trail switching (from Trail 1 to Trail 2) of ants, at some point during trailing, occurred more frequently as the disruption trail concentration increased, as shown in Fig. 4. At a base-trail concentration of 1 pg/cm, the percentage of ants that switched to the disruption trail (Trail 2; black bar) peaked when the disruption trail's concentration was 100-fold greater greater than Trail 1. Above this concentration, trails were disrupted, with few ants following either trail (grey bar). At a base-trail concentration of 100 pg/cm, the percentage of ants on the disruption trail peaked when the trail's concentration was 10-fold greater; above this, trail following was reduced substantially. The two trails appeared to merge (confounding our analysis) with greater differences between concentrations of the two trails (Fig. 1).

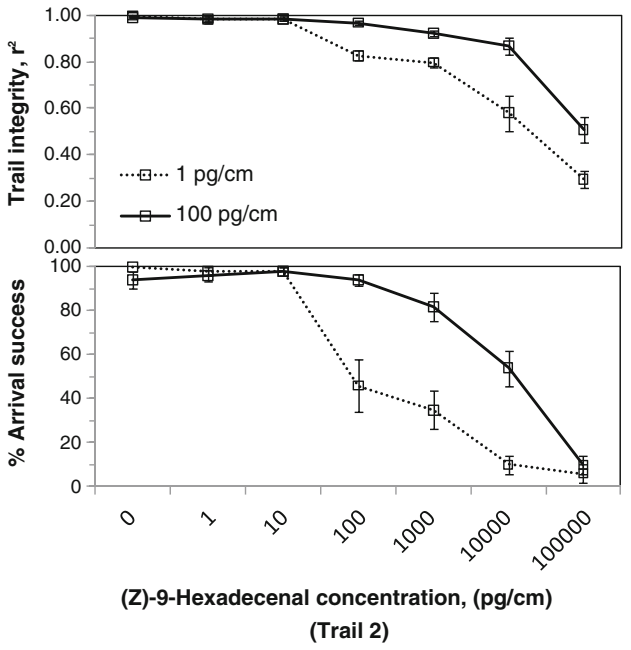


Fig. 2 Loss of Argentine ant, *Linepithema humile*, trail integrity (r^2) (*upper*) and arrival success (*lower*) over a 30 cm distance following a 1 or 100 pg/cm base trail, disrupted by an upwind trail (Trail 2) of various concentrations of synthetic (*Z*)-9-hexadecenal

Discussion

Trail following by worker ants was readily established using the trail pheromone component, *Z*9-16:Ald, over a wide range of concentrations on glass. Optimal following was in the range 1–100 pg/cm; above this, we observed a

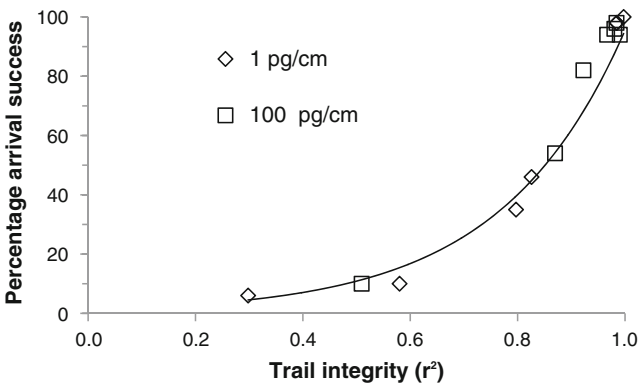


Fig. 3 Relationship between arrival success at food source and trail integrity (r^2), along base trail disrupted by an upwind trail of various concentrations of synthetic (*Z*)-9-hexadecenal, for Argentine ants, *Linepithema humile*

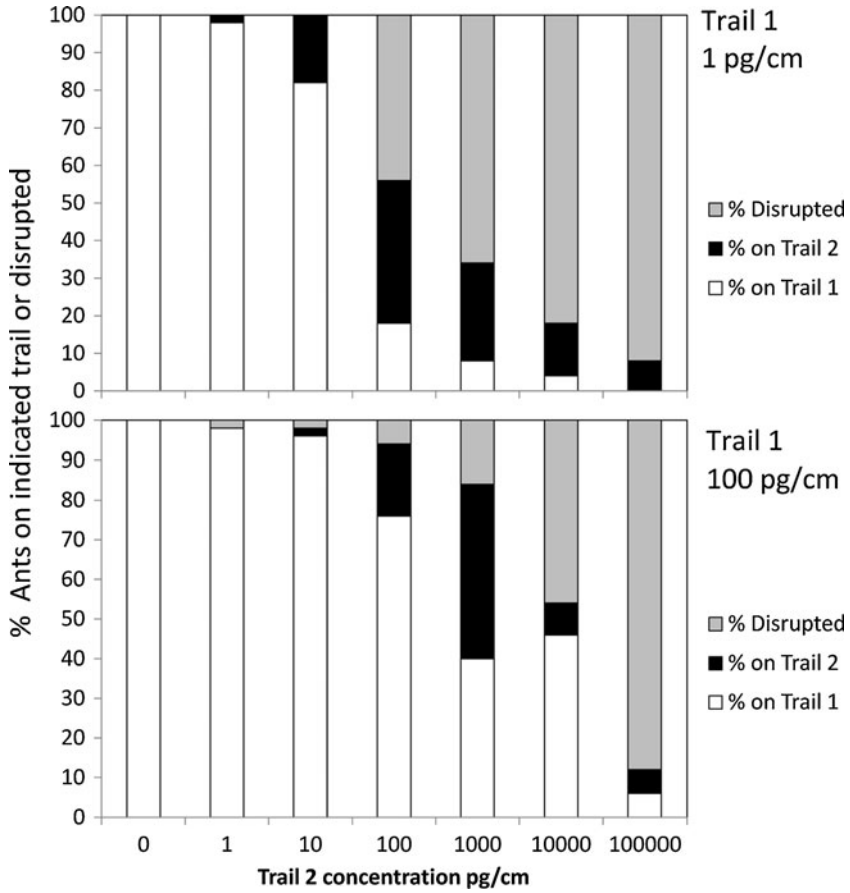


Fig. 4 Percentage of Argentine ants, *Linepithema humile*, that stayed on the base trail (Trail 1), switched to the disrupting trail (Trail 2), or were fully disrupted (not following any trail), in trials testing two concentrations of Trial 1 and various concentrations of Trial 2

decrease in trail following. This compares with the optimum amount of pheromone for trails of *Atta sexdens sexdens* (15–150 pg/cm; Morgan et al., 2006), and *Myrmica rubra* (3–300 pg/cm; Evershed et al., 1982). One pg/cm and one hundred pg/cm trails were chosen for tests of disruption, because these represented the concentration limits (i.e., weak and strong stimuli) for effective trail-following, likely reflecting low and high densities of worker ants. We demonstrated that it was possible to disrupt both trail concentrations, but only if greater pheromone concentrations were applied to the secondary (disrupting) trail. Not surprisingly, our results showed that weaker trails were more readily disrupted than stronger trails at lower concentration disrupting trails. Our trail integrity value, r^2, appeared to under-estimate trail disruption success, with a trail integrity value of 0.8 showing some impact on successful trail following, while a r^2 value of 0.6 had an arrival success of only 15%.

Hangartner (1967) manipulated trailing worker ants (*Lasius fuliginosus*) and noted that "it was observed that by asymmetric placing of the antenna tips in the scent path the animals could detect a concentration difference between left and right." Argentine ants follow trails by comparing inputs between their antennae, and move obliquely across a trail into clean air, before turning and repeating the process in a zigzag fashion. Communication disruption of trail-following ants, by excess trail pheromone in the air, could operate in several ways including, masking of the trail, sensory overload, or "false trail following". If a trail is too wide, ants can become disrupted (Morgan et al., 2006). Thus, it is possible that disruption could work by effectively widening a trail so that its boundaries with clean air are not encountered. Alternatively, sensory overload by prolonged adaptation to pheromone has been put forward as an explanation for the mechanism of disruption in a number of species of moths, including, *Epiphyas postvittana* (Bartell and Lawrence, 1973) and Oriental fruit moth, *Cydia* (=*Grapholitha molesta*) (Rumbo and Vickers, 1997), and thus, it is possible that the increased concentration of trail pheromone in the air could cause sensory adaption in the trail-pheromone ants. False-trail following, in which search effort is expended unsuccessfully, is another mechanism proposed for mating disruption in moths (Bartell, 1982). It is possible that disrupted ants lose control of their turning angles (Suckling et al., 2008) in response to instantaneous concentration peaks, and follow new trails, rather than the initial trail they were following.

Detection and response to overlapping pheromone plumes in the pea moth, *Cydia nigricana,* were studied by

Wall and Perry (1981). They found that male pea moths moved past lower dose sources for higher dose sources upwind. Later, Schofield et al. (2003) showed how almond moth *Caudra cautella* detected and responded to overlapping plumes in a wind tunnel. Morgan et al. (2006) found that workers of the ant *A. sexdens sexdens*, when given a choice of concentrations at a trail branch, always chose the more concentrated one. We observed that overstrength trails had increasing recruitment success at the expense of weaker trails. Moreover, these higher concentration trails may be reinforced by attracting more ants, and hence obtaining greater amounts of trail pheromone (Van vorhis Key and Baker, 1982b). We speculate that a similar kind of false-trail following, in response to local variation in instantaneous concentration, could cause ants to move with the tortuous and random tracks observed in this work. However, unlike the majority of moths investigated, we observed that once clean air was presented to an ant walking out of a disrupting cloud, it was able to relocate the initial trail to the food or nest. If true, such a lack of prolonged orientation disruption would mean that behavioral control of walking ants through trail pheromone disruption will be more difficult to achieve than mating disruption of flying moths.

Our results suggest greater amounts of pheromone would be needed to disrupt higher density trails, as might occur near nests and rich food sources. However, this problem may be less important if very large areas are treated over relatively long periods of time, as the disruption of new foraging trails would reduce the discovery of additional resources. Over time, this could result in a significant reduction in the flow of food back to the nest, thus reducing nest and range expansion (Aron et al., 2001).

Although these laboratory tests show promise for using this approach as a pest management system, more developmental work on suitable formulations for use in the field is needed. Moreover, we used only a single compound for trail disruption. There is some evidence for additional active compounds in the natural pheromone (Van Vorhis Key and Baker, 1982a), and identification of these could prove important for more effective trail disruption. Once a suitable formulation has been developed (e.g., Nishisue et al., 2010), larger spatial scale trials can be run for the effects of trail disruption on food foraging, and population growth in Argentine ant colonies.

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(Wyatt, 2010), which provides final assurance before allowing lambs at the udder (Lindsay, 1988). When ewes are not in parturition and not lactating, olfactory cues play an inhibitory role in maternal responsiveness, resulting in ewes displaying indifferent or hostile behavior toward neonatal lambs (Dwyer, 2008). However, for a short period, around the time of parturition, ewes become highly interested in neonatal lambs and may even steal lambs from other ewes. During parturition, odor produced by or associated with a lamb is a powerful stimulus, and neural structures such as the olfactory bulb undergo extensive changes when exposed to such odors (Kendrick et al., 1992). The bond between ewe and lamb is established through contact within 4–6 h after birth. If this fails to happen, maternal interest wanes and the ewe will not accept the lamb. However, once the bond is formed, ewe and lamb can be separated for relatively long periods without disruption of bond integrity (Lindsay, 1988).

The common factor that characterizes most post-partum deaths of lambs is impediment or disturbance of bond formation between ewes and offspring (Lindsay, 1988). In order to curtail financial losses that result from rejection of newborn lambs, a well-known phenomenon in all sheep races, farmers and scientists have examined methods of facilitating adoption of rejected lambs by ewes with ample milk. In addition to the Aristotelian method, other methods of odor transfer are practiced, albeit with limited success, by sheep farmers and have been subjected to scientific scrutiny including, for example, the transfer of the odor of a foster mother's own lamb to a rejected or orphaned lamb (Price et al., 1984, 2003; Alexander et al., 1987, 1989a, b).

Although sheep have been domesticated for about 11,000–12,000 years, and are regarded as one of the most studied animal species (Adams and McKinley, 1995), the olfactory cue that mediates ewe-lamb bonding has not yet been characterized. There are several sources from where the volatile organic compounds (VOCs) constituting a lamb's unique olfactory cue could be released. One possibility is a maternal label originating from amniotic fluid, milk, saliva, or some other secretion/excretion of the ewe. Rietdorf (2002) investigated three possible sources of sheep maternal odor: colostrum, amniotic fluid, and inguinal gland secretion. Because ewes are strongly attracted to amniotic fluid for a few hours after parturition (Lévy et al., 1983; Vince et al., 1985), farmers often smear a newborn lamb with a ewe's amniotic fluid so as to strengthen the bond (Lévy et al., 2004). However, the amniotic fluid has no effect on maternal acceptance, indicating that the fluid contains cues for general attractiveness rather than individual recognition (Lévy et al., 2004).

Alternatively, a lamb could be born with its own individual odor, with amniotic fluid having a supporting role in recognition by inducing a ewe to lick and groom a lamb, thus enabling the ewe to become acquainted with the lamb's olfactory profile. The interest that ewes display in the hindquarters, head, and neck areas of their lambs is well documented (Alexander et al., 1983; Price et al., 1984; Lynch et al., 1992; Houpt, 2005). However, from our observations, it is apparent that, in general, a ewe has no preference for a specific area on a lamb's body during the initial identification process; she simply inspects the closest part of the lamb. The lamb is finally accepted at the udder with its head turned away from the head of the ewe, at which stage the ewe, perhaps for practical reasons, shows increased interest in the lamb's hindquarters. These observations are in accord with other findings that indicate that the discrimination by ewes between their own and alien lambs is mediated primarily by olfactory cues from the lamb's wool and skin, and not from a specific area on the lamb's body or amniotic fluid (Alexander and Stevens, 1981; Lynch et al., 1992; Brennan and Kendrick, 2006). Furthermore, Kendrick et al. (1992) found that lamb odors had no influence on the activity of olfactory bulb neurons of ewes during the period before birth, but when tested after birth these odors were potent; the smell of a lamb's wool being almost as effective a stimulus as the smell of the whole lamb. Subsequently, Lévy et al. (2004) showed that ewes could recognize lambs solely through VOCs, without direct contact. Cues responsible for the individual olfactory signature of a lamb are not effective at distances greater than 250 mm (Alexander and Shillito, 1977; Alexander, 1978; Poindron et al., 2003), suggesting that the cues are either not very volatile or are present at very low concentrations.

Poindron et al. (2006) and Romeyer et al. (1993) showed that ewes use phenotype matching to recognize lambs. A ewe implanted with monozygotic twin embryos, and allowed to learn the odor of one of the twins after birth, recognized the second monozygotic lamb without being previously exposed to it. However, when this experiment was repeated with dizygotics, the ewe did not identify the twin after first being exposed to the other dizygotic (non-identical) twin. This experiment also shows that at least some features of the lamb's odor are genetically based (Wyatt, 2003).

Here, we report the results of qualitative and quantitative analyses of VOCs present in the cranial wool of Döhne Merino lambs, as well as results of preliminary field tests with synthetic mixtures of the identified VOCs.

Material and Methods

General All (borosilicate) glassware used for the collection of samples was washed thoroughly with distilled water,

heated (400°C) for at least 30 min. to remove all traces of adsorbed organics, and then cooled immediately prior to use. Samples (1 ml) of solvents dichloromethane (DCM) (Romil, Super Purity, Cambridge, UK or Pestanal Grade, Riedel de-Haën, Seelze, Germany) and *tert*-butyl methyl ether (TBME) (Sigma-Aldrich, anhydrous, 99.8%, St Louis, MO, USA) were concentrated to 10 μl in a nitrogen atmosphere (Reiter et al., 2003) and analyzed for impurities. These solvents were sufficiently pure for the extraction of semiochemicals. Syringes were cleaned by flushing barrels and rinsing plungers with DCM.

Sample Collection and Preparation A ewe flock of Döhne Merinos at Mariendahl, an experimental farm of Stellenbosch University located 14 km outside Stellenbosch, was available for study. The Döhne Merino sheep race produces a high percentage of twins. Singleton- and twin-bearing ewes were housed in separate paddocks. After birth, the singleton-bearing ewes plus lambs were moved to another paddock, while the twin-bearing ewes with lambs were moved to lambing pens for 2–3 d, after which they were housed in their own paddock. Sixteen (9.69%) of a flock of 165 twin-bearing ewes were used as a sample group. Using a pair of scissors, wool samples (*ca* 200–400 mg) were collected from the foreheads of 32 lambs on the morning after birth. Cranial wool was used because the head of a lamb should be less contaminated with foreign matter than the rest of the body. The samples were stored in glass vials (25 ml) with Teflon-faced septa at −20°C until analyzed. The wool harvested from one lamb was sufficient for several analyses (Supplementary material, Fig. S1).

The procedures that were followed in our research were approved by the Stellenbosch University Ethics Committee: Animal Care and Use (Ethics number 11NC_BU01).

Analytical Methods Gas chromatographic (GC) analyses were carried out with a Carlo Erba HRGC gas chromatograph (Milan, Italy) with a Grob split/splitless sample inlet and flame ionization detector operated at 220°C and 280°C, respectively. Gas chromatographic data were acquired with a DELTA Chromatography Data System, Version 5.0 (Digital Solutions, Brisbane, Australia). The capillary columns were manufactured by the Laboratory for Ecological Chemistry (LECUS, Stellenbosch University) and were provided with integrated retention gaps of 1–2 m. The following columns were used: column A [glass, 40 m× 0.25 mm i.d., coated with 0.25 μm of apolar PS-089-OH (DB-5 equivalent)], column B [glass, 40 m×0.25 mm i.d., coated with 0.25 μm of polar AT-1000 (FFAP equivalent)], enantioselective column C [glass, 30 m×0.3 mm i.d., coated with 0.25 μm of OV-1701-OH containing 10% heptakis(2,3-di-O-methyl-6-O-tert-butyldimethylsilyl)-β-cyclodextrin], and enantioselective column D [glass, 30 m×0.3 mm i.d., coated with 0.25 μm of OV-1701-OH containing 10% heptakis(2,3-di-O-acetyl-6-O-tert-butyldimethylsilyl)-β-cyclodextrin)]. Hydrogen was the carrier gas at a linear flow velocity of 50 $cm.sec^{-1}$ (column temperature 40°C). Samples were injected in the split mode, at a column temperature below 30°C. The column temperature was increased ballistically to 40°C, after which columns A and B were temperature programmed at 2°C.min^{-1} to 280°C and 250°C, respectively. The final temperature was held for 20 min. The enantioselective columns were programmed at 1°C.min^{-1} from 40 to 220°C.

Gas chromatographic-low resolution electron impact mass spectrometric analysis (GC-LRMS) of material from wool samples was carried out on a Carlo Erba QMD 1000 instrument (Milan, Italy), using the columns and GC parameters specified above. Electron impact (EI) mass spectral data were acquired at 70 eV from *m/z* 25–350. Helium was the carrier gas at a linear flow velocity of 28.6 $cm.sec^{-1}$ (column temperature 40°C). Inlet and interface temperatures were 220°C and 250°C, respectively. The ion source temperature was set at 180°C, and the pressure in the source housing was *ca*. 2×10^{-5} mm Hg at a column temperature of 40°C, decreasing to *ca*. 1×10^{-5} mm Hg towards the end of the temperature program. A scan rate of 0.9 $sec.scan^{-1}$ and an interscan delay of 0.1 sec were used. This instrumentation also was used for retention time comparisons of the volatiles in the wool samples (wool volatiles) with authentic synthetic reference compounds. Gas chromatography-mass spectrometry (GC-MS) was used to monitor the syntheses of reference compounds, and to confirm the identification of the wool volatiles by retention time comparison with commercially available synthetic compounds (Sigma-Aldrich, Cape Town) available from previous research projects, or compounds synthesized during the present study (Supplementary material). High resolution mass spectrometric data were acquired on a gas chromatograph coupled to a time-of-flight mass spectrometer (GC-TOF-HRMS), employing a Waters GCT Premier GC-MS instrument (Waters, Milford, MA, USA) fitted with capillary column E (30 m×0.25 mm i.d., 0.25 μm film, DB-5 ms, Agilent JW Scientific, Folsom, CA, USA). Helium was the carrier gas at 1 $ml.min^{-1}$. An inlet temperature of 260°C was used, and samples were introduced in the split mode (split ratio 1:5) at a column temperature below 30°C. The column temperature was then increased ballistically to 40°C, after which the oven was programmed at 2°C.min^{-1} from 40 to 280°C (isothermal 5 min.), followed by a 2°C.min^{-1} ramp to 300°C (isothermal for 20 min). EI-MS data were acquired at 70 eV, scanning the range *m/z* 35–600 at a rate of 0.2 sec/scan, with an inter-scan delay of 0.05 sec. The source temperature was 180°C and perfluoro-tri-*n*-butylamine was used as mass reference.

The identification of the compounds associated with the cranial wool of the experimental lambs also was substantiated by the determination of retention indices (Kovats, 1958).

^{1}H and ^{13}C NMR spectra of synthesized reference compounds were recorded in $CDCl_3$ on Varian VNMRS 300, Unity INOVA 400 and UNITY INOVA 600 NMR instruments (Varian, Palo Alto, CA, USA).

Sampling Methods

Conventional Sample Extraction Dichloromethane (DCM) and *tert*-butyl methyl ether (TBME) were tested for the extraction of wool volatiles. TBME (1 ml) was found to be more effective for extracting wool samples (300–500 mg). Methyl hexanoate (14.8 μg/ml) was added as an internal standard. Synthetic analogs of all the compounds identified also were used as external standards. Extracts were concentrated in a nitrogen atmosphere by placing a vial into a 100-ml beaker, covering the beaker with aluminum foil, and purging the solvent vapor from the beaker without directing the nitrogen flow into the vial (Reiter et al., 2003). During evaporation, the extracts were transferred to smaller Reacti-Vials. The concentration process took about 15 h to yield a final volume of *ca* 10 μl. Aliquots (1 μl) of extracts were subjected to GC-MS analysis by using split and septum purge flows of 10 and 1 $ml.min^{-1}$, respectively.

Headspace Sampling by Solid Phase Microextraction (SPME) Headspace volatiles of a sample of wool (70 mg) in a bottle (8 ml) were sampled by SPME for 15 h at 50°C in a conventional laboratory oven with air circulation.

Headspace Sampling by Sample Enrichment Probe (SEP) A second generation SEP50 sample enrichment probe (MasChrom Analisetegniek, Stellenbosch, South Africa), consisting of a fused silica stalk, or shaft, carrying a 50 mm sleeve (47 mg) of polydimethylsiloxane (PDMS) tubing (Burger et al., 2006, 2011), was used to trap VOCs from wool samples for GC and GC-MS analyses. The Teflon face of a septum was pierced centrally with the sharpened back end of the SEP, and the septum positioned on the stalk so as to enable headspace sampling from an 8-ml vial. The wool sample (70 mg) was placed in the vial, the SEP50 installed with its lower tip clearing the sample, and, after tightening the vial cap, the SEP was exposed to the headspace at 70°C for 1 h. The volatile compounds retained in the PDMS were desorbed at 220°C in the GC-MS. The SEP was left in the injector until completion of an analysis (Burger et al., 2006).

Dimethyl Disulfide Derivatization

The double bond positions of unsaturated VOCs of wool were determined by dimethyl disulfide (DMDS) derivatization and GC-MS analysis of the resulting adducts, similar to the method of Vincenti et al. (1987). An aliquot of a TBME extract of wool volatiles was concentrated to 5 μl in a Reacti-Vial, using a slow stream of purified nitrogen. The concentrate was re-dissolved in carbon disulfide (50 μl), and treated with an excess of iodine solution (5 μl of 60 mg of I_2 in 1 ml of TBME) and DMDS (50 μl). The Reacti-Vial was sealed, and the reaction mixture was heated at 60°C for 40 h. The reaction was quenched with 5% aqueous sodium thiosulfate, and the aqueous layer separated from the organic layer by centrifuging the mixture at 3,000 rpm. The organic layer was transferred to another Reacti-Vial and washed with water (2×20 μl), utilizing centrifugation to facilitate phase separation. The extract was concentrated and used for GC-MS analysis.

Determination of Absolute Configuration of Chiral Compounds

Enantioselective columns C and D were used to separate enantiomers of the chiral constituents present in wool samples.

Statistical Analysis of VOCs

Of the 133 constituents identified, only those present in 20% or more of the wool samples were considered as variables for statistical analysis, reducing the number of constituents included in the analysis to 88. The chromatographic data used for multivariate analyses still contained a large number of variables (peak areas for these identified constituents, $N \leq 88$) relative to the number of sample units (wool samples, $N \leq 32$). Peak areas were normalized across all samples to produce comparable variables with zero means and unit standard deviation (Kowalski and Bender, 1972). Permutation tests were carried out to determine whether the wool of twin lambs could be grouped according to the qualitative and quantitative composition of VOCs present in their wool. The following null-hypothesis was tested: twin lambs cannot be grouped according to the qualitative and quantitative composition of their wool volatiles. Thus, the calculated *P*-value provided an indication of whether the similarities found between twin lambs could be attributed to the fact that they are twins or to random variation in the data. Using this principle, the similarity in the odor profile of the wool of a pair of twin lambs was assessed by executing 10,000 permutation tests.

The 88 constituents and their quantities in the wool of day-old lambs born during the lambing season of 2007 were used to construct a principal component analysis (PCA) biplot. Biplots can be considered as multivariate scatterplots that simultaneously give a graphical presentation of samples (lambs) and variables (identified constituents). Lambs are displayed as points on the graph while the identified constituents are displayed as linear axes. The significance of an axis of a biplot is similar to that of an ordinary scatterplot: if a line is drawn from any point in a biplot perpendicular to a biplot axis, the value of the variable at that point can be read on the axis. In addition, the angle between any two axes approximates the correlation between two relevant variables. Constituents displayed as axes that are 90 degrees in relation to one another have no correlation with each other, and those constituents lying on axes close to one another have a high level of correlation with one another. An axis is labeled at the positive value of its calibration.

PCA biplots, permutation tests, and investigations into predictivity, were performed using R (Vienna, Austria), as described in Aldrich et al. (2004) and Gardner-Lubbe et al. (2008).

Bioassays

Bioassays were performed with singleton- and twin-bearing Döhne Merino ewes during the lambing season at Mariendahl from March to April 2009 and 2010. Singleton-bearing ewes, with their lambs, were placed in a 0.2-ha paddock within 12 h after parturition, while twin-bearing ewes, with lambs, were isolated in lambing pens (1.2× 1.6 m) inside a naturally illuminated and ventilated shed. All bioassays were conducted in these pens, outside in an enclosed concrete-surface arena (12× 8 m), or in paddocks (ca. 10,000 m^2) with natural vegetation.

Exploratory bioassays were carried out during 2009 and 2010 by dressing lambs in treated cotton fleece jackets (which served as an odor-disseminating medium), and presenting alien lambs to ewes. In order to prevent, or at least curtail, odor diffusion through the jackets during the 5 min of the bioassay, alien lambs were fitted first with disposable diapers (Huggies, New Born, Kimberly-Clark SA, South Africa). In addition, the bodies and legs of experimental lambs were wrapped in several layers of low-density polyethylene-based film (Clorox, Rockdale, Australia). Jackets were made from new, white or pale grey, fleece cloth that had been repeatedly washed in hot water and sodium dodecyl hydrogen sulfate in a domestic washing machine and air-dried on a washing line. Pieces of the cloth were subjected to SEP-headspace analysis to ensure that the material was free from contaminants that could otherwise interfere with the bioassays. Openings were made in the jackets for the legs, ears, eyes, and snouts of lambs, before placing the jackets on lambs. The jackets were fastened ventrally and under the chins of lambs by safety pins. The tail ends of some of these jackets were folded together so as to form artificial tails, and these were kept in shape with rubber bands (Fig. S2). Hoodless jackets were made with openings for the legs. These covered the body of a lamb from the buttocks to the shoulders. Some of the bioassays carried out during April 2010 were done with experimental ewes that had been conditioned to their own lambs wearing jackets. For these and other experiments requiring lambs to wear jackets for periods of up to 72 h, excess cloth was cut away from the hind legs of male lambs to prevent them from wetting jackets.

In control experiments, one of the lambs of a twin-bearing ewe was removed from the lambing pen for the duration of the experiment. The other lamb was presented to a ewe from a non-adjacent pen (so as to preclude the possibility that she could have been in occasional semi-ochemical contact with the experimental lamb). The lamb was then diapered, wrapped and presented to its own mother and then to the other ewe, after which a new cotton jacket was added, and the lamb presented again to its mother and the other ewe. Finally, it was undressed and presented to its own mother. Drastic measures were not used to facilitate acceptance of an alien lamb. An alien lamb was considered accepted if it was allowed at the udder and if the ewe directed no butts (Supplementary material, videos available at http://scholar.sun.ac.za/handle/10019.1/3980) or butt attempts toward it over a period of 5 min after first contact. If a ewe rejected an alien lamb and displayed aggressive behavior toward it, the experiment was terminated immediately and the lamb removed from the pen or experimental arena. In order to allow experimental lambs to drink, the snouts and area around the eyes were not completely covered, and thus lambs were dressed mostly in hoodless jackets. In these experiments, the lamb (N=5) was presented sideways to the ewe so that the ewe could first sniff at the jacket. After completion of the experiments, the animals were kept under observation until it was clear each lamb was not permanently rejected by its mother.

Wool extracts also were tested. Approximately 8 g of wool were sheared from the bodies of day-old lambs and Soxhlet-extracted with either pentane (N=15), diethyl ether (N=4), DCM (N=4) or ethyl acetate (N=1) for 2 h. The extracts were sprayed uniformly on new, washed fleece cloth jackets. The respective solvents were allowed to evaporate before the bioassays were carried out, always within 24 h after the wool had been harvested. In a further experiment, approximately 16 g of wool, collected from both twins of a ewe, were extracted in pentane (N=2) or DCM (N=4). To restrict co-evaporation of VOCs from the jackets, extracts were added to a freshly cleaned, new jacket in a flange flask. The flask with its contents was cooled to

−30°C, after which most of the solvent was removed under reduced pressure at the lowest temperature possible (between −30°C and −50°C), in order to restrict co-evaporation of VOCs from the treated jackets. The residual solvent traces were allowed to evaporate before the alien lambs were dressed in the jackets.

Finally, in exploratory experiments, the responses of twin-bearing ewes to alien lambs dressed in jackets sprayed with synthetic mixtures of identified wool VOCs were investigated. The synthetic mixtures were dissolved in pentane to match the composition of the wool VOCs of an experimental ewe's own lambs (analyzed as above). The bioassays were carried out within 24 h after the wool had been harvested. With the exception of a few unidentified constituents, authentic synthetic samples of all the VOCs identified in the cranial wool of the experimental lambs were available.

The interpretation of the results of the behavioral experiments was based on the consensus opinion of two or three observers.

Results and Discussion

As a working hypothesis, we presumed that the odor profiles of twin lambs were identical, or at least similar, and quantitatively different from those of other lambs. Based on the assumption that the odor of a lamb is directly related to the VOC composition in the headspace of a wool sample (i.e., to the relative percentages in which all of the compounds are present in the sample's headspace, and thus in the effluvium of the lamb), it should be possible to identify and quantify the chemicals constituting the ewe–lamb recognition cue by intra- and inter-twin comparisons of the effluvia of a representative sample of lambs. The results of GC-MS analyses, as exemplified in Fig. 1, showed that the odors in twins were indeed remarkably similar, in contrast to the differences found between the odors of lambs born to different ewes (e.g., Fig. 2).

Exploratory experiments showed that the wool of day-old lambs contained only trace quantities of VOCs, and about 5–10% of high-boiling point organics (lanolin). The minute quantities of VOCs precluded the use of solvent extraction; Soxhlet extraction and the removal of solvent from extracts was expected to result in unacceptable quantitative reproducibility and, also, the injection of large quantities of organic compounds with high boiling points could have a deleterious effect on the performance of capillary GC columns. Thus, headspace analysis was used. SPME was found to be too insensitive (results not shown). An adapted version of closed-loop stripping (Grob, 1973) also was evaluated. Although reasonable results were obtained (data not shown), this technique was rejected because the air-circulating pump became contaminated by semi-volatile compounds that could not be removed by circulating water, methanol, acetone, methanol and DCM (in that order) through the pump and purging solvent vapors from the pump by overnight pumping of purified air. Because of its high sensitivity and simplicity, the SEP technique (Burger et al., 2006, 2011) eventually was used for sample enrichment of the wool VOCs. The recently introduced term "enrichment" has gained wide acceptance, and is now preferred for sampling techniques in which the constituents of a gas sample, or the headspace of solids or liquids, are concentrated in the sample preparation step prior to introduction of the sample into the injector of the analytical instrument. A typical total ion trace (TIC) of the cranial wool volatiles of a day-old lamb is depicted in Fig. 3.

Nevertheless, several wool samples also were extracted with DCM for determination of the total composition of the collected wool and for physical data required for the

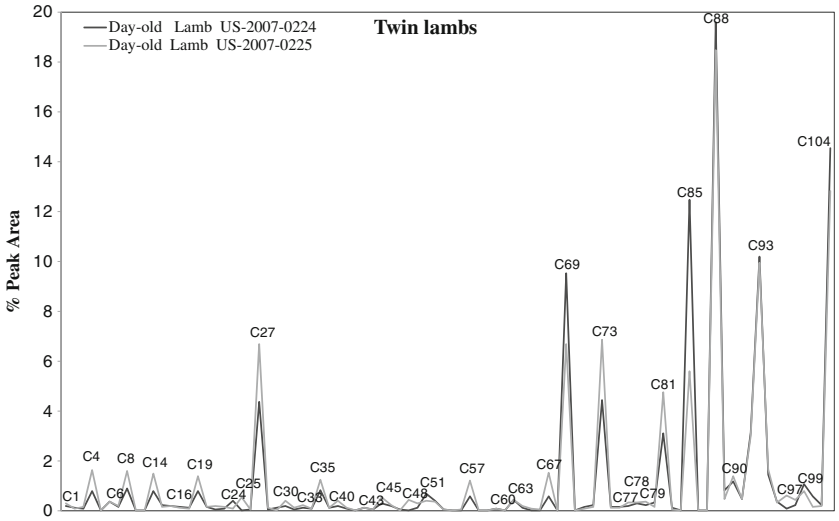


Fig. 1 Comparison of the percentage peak areas derived from mass chromatograms of the sample-enrichment-probe-enriched wool volatiles of day-old twin lambs US-2007-0224 (*black*) and US-2007-0225 (*grey*) analyzed on apolar column A. The peaks are numbered according to the information in Table 1. The minor VOCs P1–P10 were not visible in these chromatograms. Note that the concentrations of compounds 87 to 96 are so similar that the black chromatogram of lamb US-2007-0224 is only visible at the apexes of the peaks

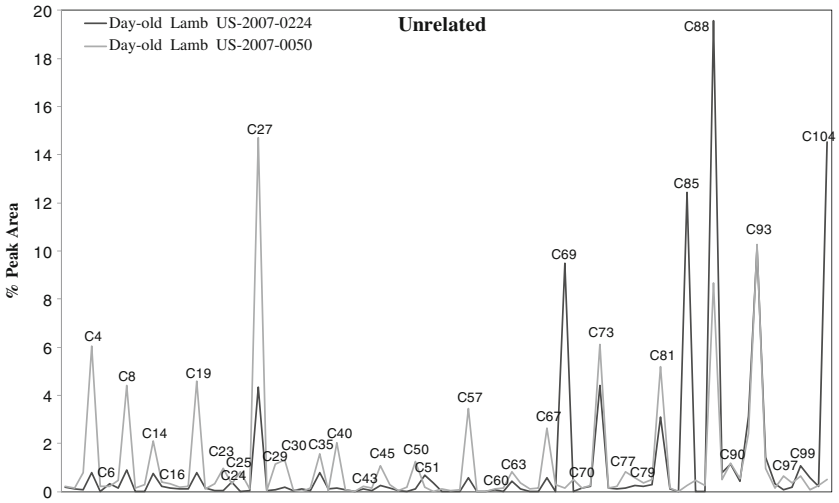


Fig. 2 Comparison of the percentage peak areas derived from mass chromatograms of the sample-enrichment-probe-enriched wool volatiles of the unrelated lambs US-2007-0224 (*black*) and US-2007-0050 (*grey*). The peaks are numbered according to Table 1

calculation of the composition of the concentration of the volatiles and semi-volatiles in the wool from the headspace data. The wool of day-old lambs typically contained 5–10% moisture, 1–4% inorganic particles, and 4–8% extractable organic material (commonly known as lanolin). The lanolin contained traces of volatile and semi-volatile organic compounds that eluted from an apolar capillary column at temperatures up to 280°C.

A total of 133 constituents (Table 1), comprising saturated and unsaturated alkanes, branched and unbranched primary alcohols, branched and unbranched aldehydes, unsaturated aldehydes, aromatic aldehydes, branched and unbranched methyl ketones, ethyl ketones, branched and unbranched carboxylic acids, unsaturated carboxylic acids, benzoic acid, esters, γ-lactones, terpenoids, 3-octanol, 2-pentylfuran, dimethyl sulfone, 3-ethyl-4-methyl-1H-pyrrole-2,5-dione, 3-ethyl-3-methyl-pyrrolidine-2,5-dione, 3-methyl-4-vinyl-1H-pyrrole-2,5-dione, and 2-ethyl-3-methylpyrrolidine-2,5-dione, were unambiguously identified by GC-LRMS and GC-HRMS analyses, Kovats retention index (RI) determination, and GC-MS comparison with authentic synthetic standards (Table 1). These compounds had masses from 60.05 to 410.39, and boiling points from 119°C to 360°C. The enantiomeric compositions of the chiral constituents [3-methyl-2-undecanone, a series of γ-substituted γ-lactones (alkan-4-olides), 3-methylpentanal and 2-ethyl-3-methylpyrrolidine-2,5-dione; Table 2] of wool were determined by GC-MS comparison with the corresponding synthetic compounds, using enantioselective columns C and D, and data published by Maas et al. (1994) and Burger et al. (2008). The terpenoids eluted as single peaks and were presumably present in the wool as pure isomers. However, since the individual enantiomers or racemates were not available for retention time comparison, it could not be established whether this assumption was correct or not. A large number of the carboxylic acids, hydrocarbons, and alcohols identified in this study have been identified previously in lanolin (Motiuk, 1979a,b, 1980; Schlossman

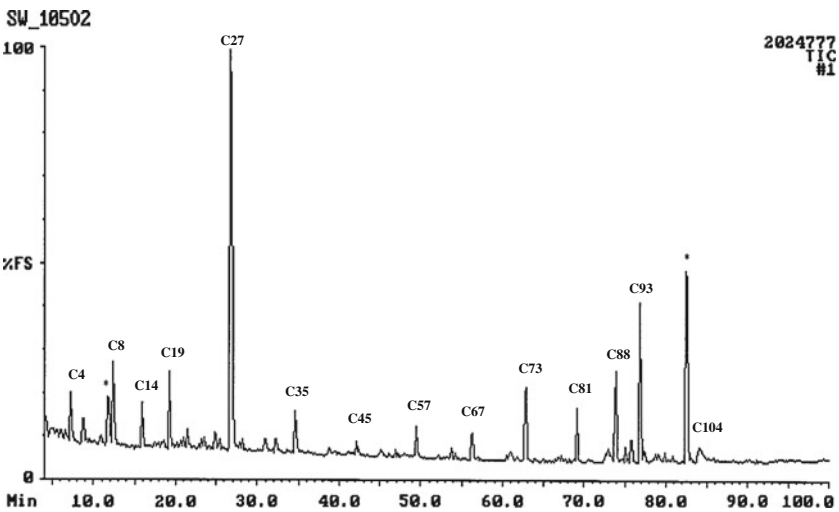


Fig. 3 Total ion chromatogram (TIC) of the headspace of the wool of Döhne Merino lamb US-2007-0105 analyzed on apolar column A. The peaks are numbered according to Table 1. Two impurities, present in all samples, are indicated by an asterisk

Table 1 Volatile organic compounds of the cranial wool of Döhne Merino lambs collected during the lambing seasons of 2007 and 2009. Constituents are grouped according to compound class

No.	Compound	Column		Remarks
		Apolar	Polar	
C10	Nonane	✓		b,c,d,k,m
C20	Decane	✓		b,c,d,k,m,o
C28	Undecane	✓		b,c,d,o
C36	Dodecane	✓	✓	b,c,d,o
C46	Tridecane	✓		b,c,d,j,o
C58	Tetradecane	✓	✓	b,c,d,j,n,o
C72	Hexadecane	✓		b,c,d,j,o
C80	Heptadecane	✓		b,c,d,j,o
C89	Octadecane	✓		b,c,d,j,o
C100	Nonadecane	✓		b,c,d,j,o
C44	1-Tridecene	✓		b,c,d,f,o
C55	1-Tetradecene	✓		b,c,d,f,m,o
C66	1-Pentadecene	✓		b,c,d,f,o
C118	1-Pentacosene	✓		b,c,e
C120	1-Hexacosene	✓		b,c,e
C2	1-Pentanol	✓		b,c,d,k
C15	1-Heptanol	✓	✓	b,c,d,o
C25	1-Octanol	✓	✓	b,c,d,j,o
C33	1-Nonanol	✓	✓	b,c,d,o
P10	1-Tridecanol		✓	b,c,d
C75	1-Tetradecanol	✓	✓	b,c,d,j,l
C87	1-Pentadecanol	✓		b,c,d,j,l,o
C98	1-Hexadecanol	✓	✓	b,c,d,j,l,m
C108	1-Octadecanol	✓		b,c,d,j,l
C114	1-Eicosanol	✓		b,c,d,j
C116	1-Heneicosanol	✓		b,c,e,j
P1	3-Methyl-1-butanol		✓	b,c,d
C6	4-Methyl-1-pentanol	✓	✓	b,c,d
P2	3-Octanol		✓	b,c,d
C4	Hexanal	✓		b,c,d,k
C8	Heptanal	✓	✓	b,c,d,k,o
C19	Octanal	✓	✓	b,c,d,o
C27	Nonanal	✓	✓	b,c,d,o
C35	Decanal	✓	✓	b,c,d,o
C45	Undecanal	✓		b,c,d,o
C57	Dodecanal	✓	✓	b,c,d,o
C67	Tridecanal	✓	✓	b,c,d,o
C73	Tetradecanal	✓	✓	b,c,d,o
C81	Pentadecanal	✓	✓	b,c,d,o
C90	Hexadecanal	✓		b,c,d,o
C101	Heptadecanal	✓		b,c,d,o
C105	Octadecanal	✓		b,c,d
C1	3-Methylpentanal	✓		b,c,d,h
C24	7-Methyloctanal	✓		b,c,d,e,m,o
C31	8-Methylnonanal	✓		b,c,d,e,o
C41	9-Methyldecanal	✓		b,c,d,e,o
C51	10-Methylundecanal	✓	✓	b,c,d,e,o
C68	12-Methyltridecanal	✓		b,c,d,e
C77	13-Methyltetradecanal	✓		b,c,d,e,o
C86	14-Methylpentadecanal	✓		b,c,d,e,m,o
C97	15-Methylhexadecanal	✓		b,c,d,e,m
C23	(*E*)-2-Octenal	✓	✓	b,c,d,e,o
C30	(*E*)-2-Nonenal	✓	✓	b,c,d,e,o
C40	(*E*)-2-Decenal	✓	✓	b,c,d,e,o
C50	(*E*)-2-Undecenal	✓	✓	b,c,d,e,f,o
C62	(*E*)-2-Dodecenal	✓		b,c,d,e,m,o
C78	(*E*)-2-Tetradecenal	✓		b,c,d,e
C13	Benzaldehyde	✓		b,c,d,n,o
C22	Phenylacetaldehyde	✓		b,c,d,n,o
C17	2-Octanone	✓	✓	b,c,d,k,o
C26	2-Nonanone	✓		b,c,d,o
C34	2-Decanone	✓	✓	b,c,d,o
C43	2-Undecanone	✓		b,c,d,o
C54	2-Dodecanone	✓	✓	b,c,d,o
C65	2-Tridecanone	✓	✓	b,c,d,o
C71	2-Tetradecanone	✓	✓	b,c,d
C79	2-Pentadecanone	✓	✓	b,c,d,o
C99	2-Heptadecanone	✓	✓	b,c,d,o
C14	6-Methyl-2-heptanone	✓	✓	b,c,d,k,o
C47	3-Methyl-2-undecanone	✓		b,c,d,e,g
C16	3-Octanone	✓		b,c,d,o
P4	3-Decanone		✓	b,c,d
P3	Acetic acid		✓	b,c,d
P5	Propanoic acid		✓	b,c,d
P6	Butanoic acid		✓	b,c,d
P7	Hexanoic acid		✓	b,c,d
P8	Heptanoic acid		✓	b,c,d
P9	Octanoic acid		✓	b,c,d,j
C42	Nonanoic acid	✓	✓	b,c,d,j
C53	Decanoic acid	✓	✓	b,c,d,j
C69	Dodecanoic acid	✓	✓	b,c,d,j,m
C85	Tetradecanoic acid	✓	✓	b,c,d,j,o
C94	Pentadecanoic acid	✓	✓	b,c,d,j,n,o
C104	Hexadecanoic acid	✓	✓	b,c,d,j,m,o
C107	Heptadecanoic acid	✓		b,c,d,j
C112	Octadecanoic acid	✓	✓	b,c,d,j
C115	Eicosanoic acid	✓	✓	b,c,d,j
C117	Heneicosanoic acid	✓		b,c,d,j
C119	Docosanoic acid	✓		b,c,d,j

Table 1 (continued)

No.	Compound	Column		Remarks
		Apolar	Polar	
C82	12-Methyltridecanoic acid	✓		b,c,d,e,j
C102	14-Methylpentadecanoic acid	✓	✓	b,c,d,e,j
C106	15-Methylhexadecanoic acid	✓		b,c,d,e,j
C113	17-Methyloctadecanoic acid	✓		b,c,d,e,j
C103	(*Z*)-9-Hexadecenoic acid	✓	✓	b,c,d,f
C110	(*Z,Z*)-9,12-Octadecadienoic acid	✓	✓	b,c,d
C111	(*Z*)-9-Octadecenoic acid	✓	✓	b,c,d,f
C32	Benzoic acid	✓	✓	b,c,d
C88	Ethyl tetradecanoate	✓	✓	b,c,d,o
C92	Isopropyl tetradecanoate	✓		b,c,d,e,o
C48	Nonan-4-olide	✓	✓	b,c,d,g,o
C61	Decan-4-olide	✓		b,c,d,g
C76	Dodecan-4-olide	✓		b,c,d,g
C109	Hexadecan-4-olide	✓		b,c,d,g
C56	6,10-Dimethyl-2-undecanone	✓		b,c,d,h
C60	(5*E*)-6,10-Dimethyl-5,9-undecadien-2-one	✓		b,c,d,o
C91	2,6,10,14-Tetramethyl-hexadecane	✓		b,c,d,h
C93	6,10,14-Trimethyl-2-pentadecanone	✓	✓	b,c,d,h,o
C95	6,10,14-Trimethyl-2-pentadecanol	✓		b,c,d,h
C122	Squalene	✓	✓	b,c,d
C5	2-Methylpyrimidine	✓	✓	b,c,d
C11	2,5-Dimethylpyrimidine	✓	✓	b,c,d
C29	*N*-Methyl-2-piperidinone	✓	✓	b,c,d
C37	3-Ethyl-4-methyl-1H-pyrrole-2,5-dione	✓	✓	b,c,i,n,o
C38	3-Ethyl-4-methylpyrrolidine-2,5-dione	✓	✓	b,i,g,n
C39	3-Methyl-4-vinyl-1H-pyrrole-2,5-dione	✓	✓	b,c,i,n
C9	Dimethyl sulfone	✓	✓	b,c,d,n,o
C18	2-Pentylfuran	✓		b,c,d,o
C124	Cholest-5-en-3β-ol	✓		b,c,d,j
C3	Unidentified	✓		
C7	Unidentified	✓		
C12	Unidentified	✓	✓	n
C21	Unidentified	✓		
C49	Unidentified	✓	✓	n,o
C52	Unidentified	✓		o
C59	Unidentified	✓		
C63	Unidentified	✓	✓	
C64	Unidentified	✓		o
C70	Unidentified	✓		o
C74	Unidentified	✓		m
C83	Unidentified	✓		m
C84	Unidentified	✓		b,m

Table 1 (continued)

No.	Compound	Column		Remarks
		Apolar	Polar	
C96	Unidentified	✓		b,c,o
C121	Unidentified	✓		
C123	Unidentified	✓		

a: Constituents identified using apolar column A are numbered from **C1** to **C124** and those identified using polar column B numbered from **P1** to **P10**

b: Low-resolution electon ionization mass spectrum

c: Library spectrum (NBS and/or NIST)

d: Retention time comparison with synthetic compound

e: Retention index

f: Double bond localization by dimethyl disulfide derivatization and gas chromatography-mass spectrometry

g: Absolute configuration given in Table 2

h: Absolute configuration not determined

i: Tentative identification

j: Compounds previously identified in lanolin (Schlossman and McCarthy, 1979; Motiuk, 1979a, 1979b, 1980)

k: Compounds previously identified in wool (Lisovac and Shooter, 2003)

l: Compounds previously identified in inguinal gland of ewes (Rietdorf, 2002)

m: Compounds identified in the wool during the lambing season of 2007 that were not identified in the lambing season of 2009

n: Compounds identified in the wool during the lambing season of 2009 that were not identified in the lambing season of 2007

o: Compounds identified in cotton fleece jacket left on lamb for 3 days

and McCarthy, 1979). In the present investigation, these compounds were not considered as potential components of the recognition cue because of their high boiling points. The constituents of such a cue must be reasonably volatile because bond formation does not require direct physical contact between the ewe and lamb (Lévy et al., 2004).

The 133 compounds identified in this study were all present in at least 10% of the samples collected during 2007. The number and varying concentrations of these compounds are consistent with the constraints on the design of chemical communication systems used in kin recognition which, according to Alberts (1992), requires a wide variety of compounds present in different concentrations. Approximately 50% of the total average peak area of the VOCs present in the headspace of the wool of the day-old Döhne Merino lambs consisted of only five compounds: ethyl tetradecanoate (20%), nonanal (12%), 6,10,14-trimethyl-2-pentadecanone (9%), tetradecanal (4%), and pentadecanal (4%).

The use of statistical analysis to elucidate active compounds in complex semiochemical mixtures is limited

Table 2 Enantiomeric composition of chiral constituents of wool determined by gas chromatography using columns C and D[a]

Compounds		Enantiomeric ratio (*R*:*S*)
C38	3-Ethyl-4-methylpyrrolidine-2, 5-dione[b]	54:46 (*N*=1, C[c])
C47	3-Methyl-2-undecanone	91:9 to 100:0 (*N*=4, C)
C48	Nonan-4-olide	69:31 to 78:22 (*N*=5, C)
C61	Decan-4-olide	58:42 to 74:26 (*N*=3, C)
C76	Undecan-4-olide	66:34 (*N*=1, C)
C109	Hexadecan-4-olide	54:46 (*N*=1, D[d])

[a] The ratios were determined by integration of single ion plots of the base peaks of the individual enantiomers. The quantitative data are given in the order of elution of the enantiomers from column C or D. In some samples, one or both of the enantiomers co-eluted with other constituents and could thus not be quantified, which accounts for the difference in the number of listed samples (*N*)

[b] Order of elution unknown

[c] Enantioselective column C

[d] Enantioselective column D

by the possibility that constituents identified as principal components in multivariate analyses might not necessarily be key components of an olfactory cue. In order to determine whether any basis existed for using twins in this study, an attempt was made to quantify intra-twin similarities and inter-twin differences in the odors of neonatal lambs by multivariate analysis of the data obtained from a randomly selected 9.69% sample of the ewe flock.

The permutation tests of the analytical data calculated a *P*-value<0.001, indicating that the pairing of twin lambs, according to the qualitative and quantitative composition of the wool, did not occur by chance, and was significant. Assuming that the odor of a lamb is related directly to the composition of the VOCs in the headspace of a wool sample, our GC-MS analyses (Figs. 2 and 3) showed that the odors of twins are remarkably similar, but there are differences between odors of lambs born to different ewes. Twin lambs thus possess odor profiles that are unique, with odor profiles more similar to each other than to those of other randomly selected non-twin lambs (See also Supplementary material, Fig. S3).

The PCA biplot depicted in Fig. 4a provides the optimal two-dimensional presentation of the data matrix. Not all 32 samples and 88 variables are equally well represented in the biplot and axis predictivity (Gardner-Lubbe et al., 2008). The quality of a biplot is an overall measure of the accuracy of the two-dimensional approximation of the data matrix and hence also of the reliability of the analytical data. Furthermore, sample predictivities and axis predictivities provide detailed information about how accurately each datum point is represented in the biplot and the degree of accuracy in the predictions made from the biplot axes. Predictivity values range from 0 to 1, with a value of 1 representing the best predictivity. In Fig. 4a, b, only the 21 axes with predictivities >0.800 are displayed. The quality of display for the PCA biplots in Fig. 4a, b is 54%, which reflects the proportion of variation in the data accounted for

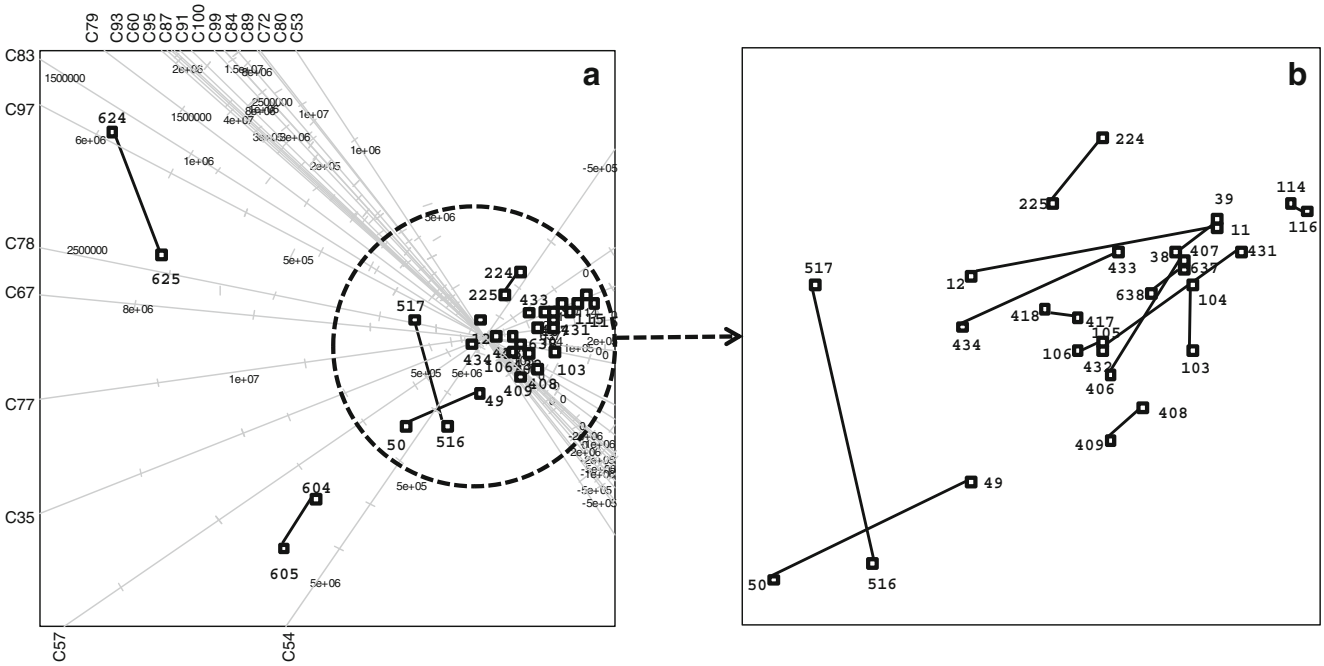


Fig. 4 **a** Principal component analysis biplot for the wool volatiles collected from day-old lambs during the lambing season of 2007; **b** PCA biplot displaying a magnified view of the crowded area in Fig. 4a. Lambs possessing similar odor profiles are circled. For identities of chemical codes refer to Table 1

in the first two dimensions of the two-dimensional display (Gower and Hand, 1996). The other 46% is explained in the remaining 30 dimensions. In Fig. 4b, the axes are not displayed in order so as to draw attention to the positions of the twin lambs. Each square represents a sample of wool collected from a day-old lamb, and the last three digits of the lamb's number are indicated in the biplot. Data pertaining to the lambs of each of the 16 twins are connected with black lines in Fig. 5a, b. The group of lambs concentrated in the encircled area indicates that these lambs possess odor profiles that are generally similar. The fact that non-twins (e.g., lambs 12 and 434) are closer in Fig. 4a, b than some twins can perhaps be explained in terms of possible variables that were not chosen in this experiment.

The 21 constituents with predictivities>0.800 in order of decreasing predictivity are 2,6,10,14-tetramethylhexadecane (**C91**), 2-pentadecanone (**C79**), octadecane (**C89**), 1-pentadecanol (**C87**), (5*E*)-6,10-dimethyl-5,9-undecadien-2-one (**C60**), 15-methylhexadecanal (**C97**), unidentified constituent **C84**, (*E*)-2-tetradecenal (**C78**), 2-dodecanone (**C54**), tridecanal (**C67**), decanoic acid (**C53**), decanal (**C35**), 6,10,14-trimethyl-2-pentadecanol (**C95**), 13-methyltetradecanal (**C77**), hexadecane (**C72**), heptadecane (**C80**), nonadecane (**C100**), 2-heptadecanone (**C99**), unidentified constituent **C83**, 6,10,14-trimethyl-2-pentadecanone (**C93**) and dodecanal (**C57**). Interestingly, all the aldehydes have maxima on the left-hand side of the plot. All the methyl ketones, except 2-dodecanone, and all the hydrocarbons and decanoic acid are highly correlated with each other.

In 2009, eight, day-old twins from five of the ewes that belonged to the sample group in 2007, were available for further investigation. These twins and those used in 2007 were sired by different rams. As for the wool volatiles collected in 2007, in 2009 only the volatiles present in 20% or more of the samples collected were considered as variables for statistical analysis, which reduced the number of variables to 84 constituents. The similarity in the odor profiles of the wool of a pair of twin lambs was again assessed by executing 10,000 permutation tests. A *P*-value of 0.006 was calculated, indicating that the null hypothesis was rejected, and that the twin lambs born in 2009 possessed odor profiles that were unique and more similar than those of randomly selected non-twins. A higher *P*-value was obtained for lambs investigated in 2009 than for those investigated in 2007, but this could be attributed to the smaller number of wool samples available in 2009.

The flock of ewes used in this project is being used for a long-term sheep breeding study at the Faculty of AgriSciences, and thus we could not carry out an experiment examining whether wool volatiles of offspring produced by the same parents are quantitatively and qualitatively similar from one lambing season to the next. A comparison of wool volatiles from lambs born in 2007 and 2009 from the same ewes, but with different fathers, showed that odor profiles of the 2 years were not different, with nearly all the constituents identified in wool in 2007 also present in 2009. However, the PCA biplot (Fig. 5) shows a definite separation between wool volatiles from lambs born in 2007 and those born in 2009, indicating substantial quantitative differences. This provides evidence that the odor profiles of lambs born of the same ewe, but sired by different rams, are not identical. We are studying this aspect in more detail.

The quality of display of the PCA biplot (only the 20 constituents with predictivities >0.800 are displayed) in Fig. 5 is 56%. The constituents, in order of decreasing predictivity, are tridecane (**C46**), 13-methyltetradecanal (**C77**), 3-octanone (**C16**), 1-heptanol (**C15**), hexanal (**C4**), unidentified constituent **C64**, tridecanal (**C67**), (*E*)-2-tetradecenal (**C78**), octanal (**C19**), (*E*)-2-nonenal (**C30**), 2-pentadecanone (**C79**), tetradecanal (**C73**), dodecanal (**C57**), hexadecane (**C72**), 6-methyl-2-heptanone (**C14**), (*E*)-2-octenal (**C23**), heptanal (**C8**), *N*-methyl-2-piperidinone (**C29**), hexadecanal (**C90**) and 2,5-dimethylpyrimidine (**C11**). It is clear from Fig. 5 that lambs born in 2007 have larger quantities of all these compounds. The figure suggests that the two nitrogen containing constituents, *N*-methyl-2-piperidinone (**C29**) and 2,5-dimethylpyrimidine (**C11**), are highly correlated. All the hydrocarbons, ketones

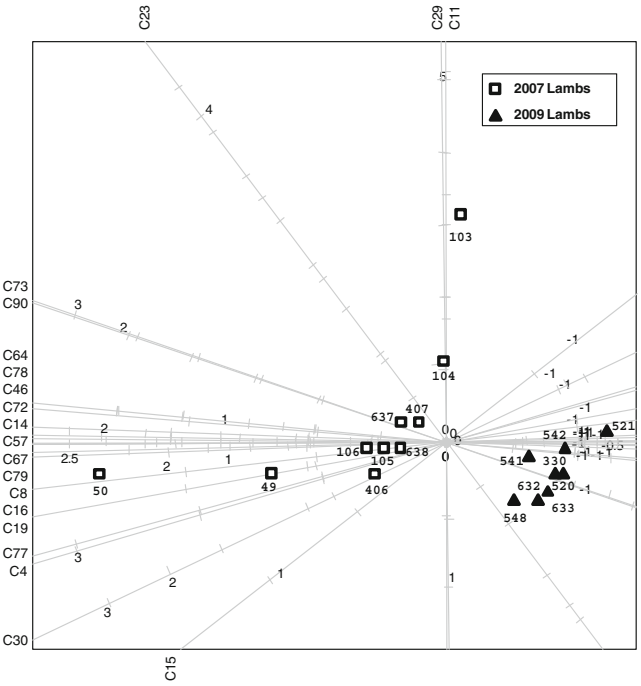


Fig. 5 Principal component analysis biplot for the wool volatiles collected from day-old lambs during the lambing seasons of 2007 and 2009. For identities of chemical codes refer to Table 1

and aldehydes, except (*E*)-2-octenal (**C23**), also correlate reasonably well with each other.

Price et al. (1984), placed nylon stockinettes worn by lambs for 72 h on alien lambs, which were then presented to ewes. Initially, 38% of alien lambs were accepted immediately and another 50% accepted over a further 36 h. More drastic measures have been used in other studies to force ewes to accept alien lambs treated with various odors or synthetic materials. For example, ewes were tethered to prevent them from displaying their typical udder denial behavior in experiments that continued over periods of up to 72 h (Alexander et al., 1987, 1989b).

We carried out similar experiments. However, the experimental lambs were diapered and wrapped in polyethylene film to impede the diffusion of odor of the lambs into the treated jackets. In our control experiments, carried out to test the effect of diapers and polyethylene film, the majority of ewes rejected the wrapped and dressed alien lambs (Table 3). A smaller majority of ewes rejected their own wrapped and dressed lambs. Although the lambs did not bleat much, it is possible that these ewes could have recognized their own lambs by auditory or visual cues. It was apparent, nevertheless, that the diapers plus polyethylene film were sufficiently impermeable to restrict diffusion of wool volatiles into the cotton jackets during the short bioassays (ca 5 min).

The results in Table 3 draw attention to the problems involved in the interpretation of various behavioral responses of ewes when approached by an alien lamb. The mothering instinct of ewes varies: some tolerate the presence of an alien lamb as long as it keeps its distance, while others react aggressively, butting the lamb. The behavior of ewes also varies depending on the number of lambs they have mothered. Young ewes are normally more suspicious and more aggressive than more experienced ewes, perhaps because older ewes have become accustomed to the presence of observers. In bioassays, typical behavioral patterns of ewes were observed, ranging from total rejection to enthusiastic acceptance, including, immediate and repeated aggressive butting of the lamb, one or more less aggressive buts, moving in circles to prevent the lamb from getting at the udder, stamping of a hind foot on the side of the approaching lamb, stepping gently aside, allowing the lamb to approach, allowing the lamb to drink for a few moments before moving away or butting it, allowing the lamb to drink for longer periods before moving away or, finally, accepting the lamb and uttering low grunts of acceptance and/or encouragement. The reaction of the ewe also depended on how thirsty the lamb was, or on or how persistent it was at getting to the udder. It was clear that some lambs smelled or in some other way realized that an experimental ewe was not its mother. In some cases, the ewe and alien lamb simply avoided each

Table 3 Efficacy of diapering and wrapping experimental lambs in polyethylene foil in order to curtail the release of the recognition cue during bioassays[a]

	Alien lamb Naked	Own lamb Diapered and wrapped	Alien lamb	Own lamb Diapered, wrapped and jacketed	Alien lamb	Own lamb Naked afterwards
Ewe 79 Lamb 298	Rejected[b]	Rejected	Rejected without being sniffed at	Rejected	Rejected	No sign of rejection; lamb did not attempt to drink[c]
Ewe 298 Lamb 79	Avoided by ewe	Rejected; did not attempt to drink	Rejected	Rejected	Rejected	Accepted immediately[d]
Ewe 204 Lamb 96	Avoided by ewe	Accepted at the udder, then rejected	Did not attempt to drink; ewe showed no aggression	Ewe avoided lamb; no aggression	Did not attempt to drink	No sign of rejection; lamb did not attempt to drink
Ewe 188 Lamb 78	Rejected	Accepted at the udder	Rejected	Accepted at the udder	Rejected	Accepted immediately
Ewe 78 Lamb 188	Avoided by ewe	Accepted at the udder	Avoided ewe; did not attempt to drink	Avoided by ewe; did not attempt to drink	Ewe avioded lamb	Accepted immediately
Totals[e]	Rejected 100%	Rejected 60%	Rejected 80%	Rejected 80%	Rejected 80%	Accepted 100%

[a] The experiments were carried out in the absence of the other twin of the twin-bearing ewes. The wrapping and dressing procedure is described in the text

[b] Based on butting of the lamb and/or various ways of avoiding contact with the lamb

[c] Probably due to the lamb being awkwardly dressed

[d] Immediately accepted at the udder; in some cases accompanied by ewe's typical low grunts encouraging the lamb to drink

[e] Based on consensus of opinion of two observers

other, a situation that made it difficult to decide whether the ewe rejected the lamb or *vice versa*. A small minority of ewes did not accept any lamb. In another set of control experiments, we observed a ewe aggressively butting a jacketed alien lamb without having sniffed at it and afterwards even rejecting her own twin lambs for more than an hour.

In further experiments carried out in 10,000-m^2 paddocks, lambs of singleton-bearing ewes wore hoodless cotton fleece jackets for 72 h. Alien lambs, dressed in these jackets and presented to the respective ewes, were all accepted by ewes, without any of the typical signs of rejection (acceptance rate 100%; N=6). The experimental lambs then were undressed and returned to their mothers and were all immediately accepted. One of these jackets was stored in a glass jar and the VOCs in the headspace analyzed by GC-MS. Not all the previously identified VOCs were detected (Table 1), probably because of partial evaporation of the more volatile constituents during the bioassay. On the other hand, no compounds other than those listed in Table 1 were detected.

Because our qualitative chemical analyses were carried out on lambs born to twin-bearing ewes, we conducted similar bioassays with the twin-bearing ewes. An acceptance rate of lambs of only 62% (N=13) was obtained. The difference in acceptance rates between singleton- and twin-bearing ewes could possibly be ascribed to increased stress on the twin-bearing ewes due to the experiments being carried out in a concrete arena or in small lambing pens in a shed housing about 40 other ewes with twins (as opposed to the paddocks for the singleton-ewe trial). Alternatively, the absence of the second twin during the experiment may also have contributed to the stress of these ewes.

Similar experiments, spraying extracts of 8 g of lamb wool on new washed fleece cloth jackets were carried out. In five (20%; pentane, N=4; dichloromethane, N=1) of the experiments, the alien lambs were accepted by ewes. Although the small sample size precluded meaningful statistical analysis, we suspect that selective co-evaporation of some of the more volatile compounds with the solvent could have contributed to the low acceptance rate. Headspace analyses of jackets treated with these wool extracts, or with mixtures of synthetic VOCs, revealed that VOCs are not released from the treated jackets in the same ratios in which they are present in the wool or in the synthetic mixtures. These results showed that the volatile, apolar compounds, in particular, are prone to co-evaporation with the solvent. It is also possible that the dissemination of VOCs from cotton cloth differed from that of lamb's wool, or that some constituents were converted to other products during Soxhlet extraction.

A more concentrated extract of 16 g (collected from both twins of the ewes) of wool was applied to the jackets, with solvent evaporated at low temperature so as to minimize the co-evaporation of VOCs. Ewes (N=6) rejected all the treated alien lambs, although they did sniff at the jackets for longer than in previous bioassays before rejecting the aliens.

Finally, twin-bearing ewes rejected all (N=4) the alien lambs dressed in jackets sprayed with mixtures of synthetic VOCs. With the exception of a few unidentified constituents, authentic synthetic samples of all the VOCs identified in the cranial wool of lambs were available. Therefore, for these experiments we did not have to select only the 21 constituents with predictivities >0.800, as in the statistical analyses of our analytical results.

Our observations in these experiments suggested that cues other than lamb odor might be used by ewes when confronted with a decision of whether to accept or reject a lamb. In the experiments we could not preclude alien lambs from bleating, nor could we cover the heads and snouts of the lambs in such a way that they could still see and drink while preventing ewes from finding unprotected parts of the lambs to sniff at. The jackets appeared to hamper the normal persistence of lambs to get at the udder, and the awkward gait of lambs dressed in this manner could have triggered their rejection.

Future work will focus on finding a more reliable bioassay, and on devising methods for dissemination of the VOCs in natural concentrations and ratios.

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2006). This predator is known to aggregate on trees infested by bark beetles, especially during the earliest stages of colonization (Dixon and Payne, 1979). Adult *T. dubius* locate trees under colonization by bark beetles by using bark beetle pheromone components such as frontalin (*D. frontalis*), ipsenol (*I. grandicollis*), and ipsdienol [*I. pini, Ips calligraphus* (Germar), and *Ips avulsus* (Eichhoff)] that act as kairomones to guide them to their prey. Each kairomonal signal can be perceived alone (Payne et al., 1984; Herms et al., 1991; Raffa, 2001) or associated with tree-produced monoterpenes such as α- and β-pinene (Billings and Cameron, 1984; Erbilgin et al., 2003; Seybold et al., 2006; Reeve et al., 2009). This predator also responds to sulcatol in the field (Costa and Reeve, unpublished data), a pheromone component emitted by the ambrosia beetle *P. flavicornis* (Renwick et al., 1977).

Although *T. dubius* can respond to a broad range of volatiles, including kairomonal signals, little is known about the olfactory behavior of individuals. Previous studies have concentrated on field trapping (Billings and Cameron, 1984; Strom et al., 1999; Erbilgin and Raffa, 2001; Erbilgin et al., 2003; Reeve et al., 2009) or wind tunnels with groups of predators (Mizell et al., 1984), whereas individuals have only been investigated with electroantennography (e.g., Payne et al., 1984). Understanding predator preference at the individual level is important for understanding the mechanisms that underlie responses of *T. dubius* populations in the field. For example, is each individual predator a generalist able to respond toward a broad range of semiochemicals (e.g., kairomones), or do predator populations consist of groups of individuals with fixed prey preferences and the capacity only to respond to specific semiochemicals? When differences among populations in kairomone preference are observed (Reeve et al., 2009), is this generated by changes in individual behavior or by the relative abundance of various specialists (chemomorphs)? To date, these questions have not been addressed, but could be examined by using wind tunnel observations (Costa and Reeve, 2011). In particular, *T. dubius* displays a typical antennal behavior prior to upwind flight in response to prey pheromones, to tree volatiles, or to a mixture of both (Costa and Reeve, 2011). This methodology could be used to test for the occurrence of chemomorphs in *T. dubius* populations.

Because bark beetle populations can greatly fluctuate in both abundance and species diversity (Turchin et al., 1991; Reeve and Turchin, 2002), generalist predators like *T. dubius* may have evolved a number of strategies to survive despite unpredictable prey resources. The prolonged developmental time and diapause plasticity of *T. dubius* (Reeve, 2000) might limit the detrimental effects caused by extreme prey fluctuations on their survival. These generalist predators also may benefit from altering their preference for different prey species through switching behaviors (Murdoch, 1969; Křivan, 1996). Field-trapping studies suggest that *T. dubius* populations became more responsive to *Ips* pheromones when *D. frontalis* is absent or endemic, in a pattern that could be generated by switching behavior (Reeve et al., 2009). If switching does occur, it may imply that an individual can learn cues associated with the most abundant prey, thereby increasing its foraging efficiency (Murdoch, 1969; Mizell et al., 1984; Turlings et al., 1993). Optimal foraging theory predicts that learning should increase fitness by maximizing a forager's ability to exploit unpredictably distributed resources (Stephens and Krebs, 1986; Křivan, 1996).

Learning can be defined as a modification of behavior through experience (Papaj and Prokopy, 1989). In many studies that have investigated the effect of olfactory experience among insects, associative learning (*sensu* Pavlov, 1941) has been demonstrated to increase the response to an initially low or non-attractive stimulus after a paired exposure of this stimulus with a reward (Vet et al., 1995; Cunningham et al., 2004; Chaffiol et al., 2005). Finally, simple exposure to a stimulus also could enhance the response by sensitization (*sensu* Smith, 1993). The effect of olfactory experience on the chemo-orientated response of true predators is largely unexplored in arthropods (De Boer and Dicke, 2006; Dukas, 2008), with limited evidence from anthocorid bugs (Drukker et al., 2000a), and predatory mites (Drukker et al., 2000b). *Thanasimus dubius* could be a good candidate to learn cues from its environment (Mizell et al., 1984; Reeve et al., 2009) because of its broad potential diet, the unpredictable distribution of its prey resources in both space and time, and its long-lived adult stage. Although *T. dubius* might learn to recognize tree volatiles as well, prey pheromones would seem a more reliable indicator of the presence of a particular prey (Vet and Dicke, 1992).

We conducted three experiments to investigate the ability of individual *T. dubius* to respond to different prey olfactory signals (bark beetle pheromone components) and to assess this predator's potential for learning. Although there is substantial literature on the responses of *T. dubius* to prey pheromone components, these studies evaluated the response of predator populations and could mask variation among individual responses. Our study combines field data and wind tunnel responses of individual *T. dubius* in order to understand how these predators can cope with fluctuations of prey resources. To our knowledge, this is the first evidence that the behavior of a bark beetle predator could be influenced by previous olfactory experience.

Methods and Materials

Collection and Handling of Adult Thanasimus dubius (Wild Individuals) Wild adult *T. dubius* used in Experiment 2 (see

below) were captured in central Wisconsin at Black River Falls State Forest (Millston, WI, USA) with 12-unit funnel traps (Lindgren, 1983) (Contech Enterprises Inc., Delta, BC, Canada) baited with frontalin and turpentine. Racemic frontalin was released from two 0.4 ml polyethylene vials, each loaded with 0.3 ml (297 mg) of frontalin to achieve a release rate of 5.2 mg/d at 20°C (Contech Enterprises). A 40 ml volume of turpentine (EZ Gum Turpentine, E. E. Zimmerman Co., Pittsburgh, PA, USA) was released from a 30 cm long piece of polyethylene tubing sealed at both ends, which produced a release rate of 1 g/d at 20°C (Reeve et al., 2009). Field-captured *T. dubius* were brought to the laboratory in plastic bags (17 cm × 24 cm) inside a cooler and stored for<10 d at 8°C with most insects being kept< 7 d. Four days before use, they were placed by groups of 40–50 individuals in cylindrical plastic containers (21 cm high × 21 cm diam) and held (no longer than 14 d) in an incubator at 23.0±0.5°C and a photoperiod of 16 L/8D until testing in the wind tunnel. All individual *T. dubius* were fed with adults of the cowpea weevil, *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchidae), a factitious prey (Nebeker et al., 1980; Reeve et al., 2003). Water was provided with a wet piece of sponge. On the day before the test, wild *T. dubius* were placed individually in labeled plastic Petri dishes (9 cm diam) where they were provided with a wet sponge and fed *C. maculatus* (6–8 prey/individual).

Rearing of Adult Thanasimus dubius (Naive Individuals) All predators used in Experiment 3 (see below) were produced by this rearing method. The laboratory culture was initiated with wild *T. dubius* collected in Louisiana in 2004 by using 12-unit funnel traps baited with frontalin and turpentine with the same baits and release rates as mentioned above. Insects were reared through at least two generations in the laboratory. Adults were held, fed, and watered as described above. Eggs of *T. dubius* were collected on folded paper towels. Larvae were fed a meridic diet that is a mixture of foodstuffs and other ingredients that have been used to rear *T. dubius* (Reeve et al., 2003). This diet was presented in Parafilm® capsules, with each capsule containing approximately 0.3 ml of diet. To avoid cannibalism, newly hatched larvae were kept individually in 50 mm Gelman Petri dishes (Pall Life Sciences, Port Washington, NY, USA) covered with a Kimwipe® tissue. Each larva was provided with one or two Parafilm® capsules of diet and a moistened sponge every 2–3 d. When the larvae turned purple, indicating a readiness to pupate, they were provided with a piece of sterilized pine bark. After the larvae entered the bark, the bark pieces were placed together in 17 × 12 × 6 cm plastic containers. Emergence was examined every 2 d so that adult *T. dubius* could be separated into groups of similar age.

All adult *T. dubius* used in Experiment 3 were thus considered semiochemically naive because (1) larvae were reared on artificial diet; (2) adults were fed factitious prey; and (3) adults were not exposed to any semiochemical before testing them in the wind tunnel.

Wind Tunnel Flight Bioassays Our wind tunnel, similar to that of Visser (1976), consisted of a 140 × 70 × 70 cm Plexiglas flight chamber. Air flow was created by using a box fan. Between the chamber and the fan, a 2 cm thick activated charcoal filter was set to purify incoming air, followed by four layers of cheesecloth mounted on a wooden frame to make the airflow laminar. The flight chamber was illuminated by six 120 cm long 58 W fluorescent tubes placed on top of the chamber. To limit phototaxis created by intense light, a Plexiglas light diffuser and a white cotton sheet were interposed between the lights and the top of the chamber. A platform, made of a clear plastic cylinder (14 cm high × 14 cm diam), was used to release insects and was placed 80 cm downwind from the source. Airspeed at the release point was 0.1 m/s. Low-speed designs have been used previously with success for other Coleoptera (Salom and McLean, 1990; Wyatt et al., 1993; Heath et al., 2001); stronger winds can inhibit upwind flight of some insects (Mizell et al., 1984; Marchand and McNeil, 2000). Air exited the tunnel into a fume hood and was exhausted from the building. Temperature, humidity, and barometric pressure were recorded in the room during testing. Air in the tunnel was humidified to ensure that it remained above 50%. As the humidifier tended to cool the air, a small space heater was used to match temperature in the chamber with the room. Room temperature was always 23±1°C. To reduce any potential bias created because of observer presence or other visual stimuli, the tunnel was covered with a white sheet during the test. Behavior of *T. dubius* was observed through two small apertures in the sheet.

Tests were always conducted between 1300 and 1800 h. This time remained constant throughout the entire experiment to limit any potential effect of time on beetle response. Each insect was tested individually. For each test, the odor source was first introduced into the wind tunnel for 1 min; then the insect was released on a 3 × 3 cm section of paper towel on the platform center. Behavior was monitored for 5 min or until the insect left the platform either by flying or walking, although walking seldom occurred in preliminary tests. In all experiments, the platform was located 80 cm downwind from the odor source. We used the same methods as in Costa and Reeve (2011).

We have shown previously that adult *T. dubius* display a typical "stand up" antennal behavior (where the insect makes a complete stop, erects its antennae, and while

elevating the thorax lifts one of its front legs) before flying upwind, and that significantly more stand up antennal behaviors are performed when exposed to an attractive source (Costa and Reeve, 2011). We further designated as "upwind flights" those flights in which the insect landed upwind *and* displayed at least one stand up antennal behavior, because this appears to indicate a genuine olfactory response to the chemical source.

The total number of stand up antennal behaviors was recorded, and their mean rate (number of behaviors/individual/min) was calculated for each treatment. Time until takeoff and the landing position also were recorded. Lines drawn every 10 cm on the tunnel floor were used to indicate the landing position as a distance from the odor source. In both Experiments 2 and 3, we used the number of insects flying upwind to compare the attractiveness of each treatment. In Experiment 3, we also present for each treatment the mean rate of stand up antennal behaviors (number of behaviors/individual/min).

Experiment 1: Mark-Release-Recapture and Field Choice Test with Wild Thanasimus dubius In 2006 and 2007, we examined the responses of individual *T. dubius* adults to three prey pheromone components (frontalin, ipsenol, and ipsdienol), by using a combined mark-release-recapture experiment and field choice test. At each site, 12-unit funnel traps were arranged in an 86 m diam ring consisting of nine traps, set 30 m apart. Rings of traps were all located in red pine, *Pinus resinosa* Ait., plantations that had similar densities (trees were 6–8 m apart from each other), and the traps were intermingled among the trees. Within the same ring, traps were baited with the three prey pheromones (racemic ipsenol, ipsdienol, and frontalin, Table 1), providing three replicates for each semiochemical (Fig. 1). The sequence of prey pheromones was determined randomly for each ring. We set up a total of five rings at two sites: two at Black River Falls State Forest (Millston, WI, USA) and three at Dell Creek Wildlife area (Reedsburg, WI, USA). Because two of the rings at Dell Creek Wildlife area were both used twice, in 2006 and 2007, we obtained a total of seven blocks for the whole experiment. Insects that responded to one compound (i.e., captured in a trap baited with that prey pheromone) were stored at 8°C (less than 10 d), and then released from a piece of burlap attached to a tree located in the center of a ring. Predators captured with a particular pheromone were color-coded (Fig. 1) by applying enamel paint to the pronotum (Cronin et al., 2000). We then recorded the subsequent prey pheromone choices of the marked individuals within the trap ring.

Experiment 2: Breadth of Kairomonal Range in Wild Thanasimus dubius Our field experiment (Experiment 1) suggested that individual *T. dubius* were attracted to the three prey pheromone components. We then used a wind tunnel to investigate the extent to which this behavior may occur for a wider range of semiochemicals.

All predators used in this experiment were wild predators, collected in Wisconsin (see above). Each predator was tested twice in the wind tunnel described above (see *Wind Tunnel Flight Bioassays*), with two of the following stimulus sources: frontalin, ipsenol, ipsdienol, sulcatol, and α-pinene. The two sources were presented the same day, within a 2 h interval. Although some predators were tested with the same source twice (e.g., frontalin-frontalin), others were tested with two different sources (e.g., ipsenol-sulcatol). We also compared all semiochemical treatments to a control group, where all individuals were tested during both trials with no chemical (blank). A total of N=220 individual *T. dubius* were tested. We tested N=40 insects for each semiochemical treatment during the first trial (thus, a total of N=40 x 5=200) and N=20 were used for the control. In the second trial, each group of 40 insects tested toward a semiochemical was then divided equally into 5 groups. The 20 individuals tested to the blank during the first trial were all retested with a blank so that each trial could be compared to a "no chemical source" situation with the same level of testing in the wind tunnel. Frontalin, ipsenol, and ipsdienol (all racemic) were released at 1–2 mg/d (20°C), whereas

Table 1 Background data on semiochemicals used in flight experiments with *Thanasimus dubius*, 2006–2007

Semiochemical[a]	Elution device	Chem. Purity	Release rate	Vendor	Used in
Frontalin[b]	Polyethylene vials (0.25 ml)	99%	0.5–1.0 mg/d	Contech	Experiments 1, 2, & 3
Ipsenol[b,c]	Bubble caps	98%	0.5–1.0 mg/d	Contech	Experiments 1, 2, & 3
Ipsdienol[b,c]	Bubble caps	98%	0.5–1.0 mg/d	Contech	Experiments 1, 2, & 3
Sulcatol[c]	Bubble caps	98%	2.2 mg/d	Contech	Experiment 2
α-Pinene	Polyethylene transfer pipette Samco # 252	98%	100 mg/d	Sigma Aldrich	Experiment 2

[a] All semiochemicals used were racemic

[b] Two bubble caps or vials were used in Experiment 2 to achieve a 1.0–2.0 mg/d release rate

[c] Semiochemicals formulated in bubble caps were diluted in the inert solvent acetyl tributyl citrate to prevent polymerization

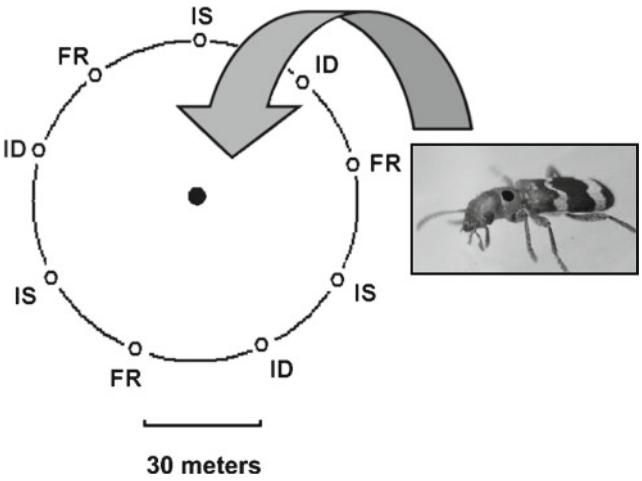


Fig. 1 Experimental design used for the mark-release-recapture experiment: marked *Thanasimus dubius* were released at the center of a ring of multiple-funnel traps baited alternately with prey pheromones: FR = frontalin, IS = ipsenol, and ID = ipsdienol (*T. dubius* photo: M. Venarsky and A. Costa)

sulcatol was released at 2.2 mg/d (20°C) (Contech Enterprises). Racemic α-pinene (98% chemical purity, Sigma-Aldrich Chemical Company Inc., St. Louis, MO, USA) was released at 100 mg/d (20°C) from a Samco #252 clear pipette (Samco Scientific, San Fernando, CA, USA). We used these release rates because: (1) they are known to be active in field experiments (Erbilgin and Raffa, 2001; Reeve et al., 2009) and previous wind tunnel studies (Costa and Reeve, 2011); and (2) we were primarily interested in examining the effect of experience rather than different concentrations on attraction.

Experiment 3: Effect of Experience on Thanasimus dubius Response Because individual *T. dubius* showed an ability to respond to different semiochemicals in Experiments 1 and 2, suggesting that they are true generalist predators, we also studied the ability of *T. dubius* adults to learn cues from their environment. All *T. dubius* used in this experiment were initially naive (see *Rearing of Adult Thanasimus dubius*).

We examined two forms of experience: sensitization and associative learning. Some treatments *exposed* adult *T. dubius* twice to one prey pheromone component, whereas other treatments *conditioned* them to a prey pheromone component by exposing the individual to the component when the beetle was consuming a food reward. As a reward, we used adult *C. maculatus*, which is a prey that is not likely associated with any bark beetle pheromone components. We also included naive groups of predators that were *well-fed* (*ad libitum*) to ensure that starvation would not modify predator responsiveness to a particular prey pheromone component. We recorded flight responses toward two major prey pheromone components, frontalin and ipsenol, presented alone or associated with the reward. Thus, we compared the percentages of upwind flights and the mean rate of stand up antennal behavior between predators, initially naive, that either received some type of olfactory experience (*exposed* or *conditioned*), or stayed naive (*control* groups).

Prior to testing, naive adult *T. dubius* were isolated in Petri dishes as in the first wind tunnel experiment and starved for 10 d. This starvation period ensured that the predators would readily attack a prey item. Just prior to exposure, all insects were placed into conditioning dishes made of a 9 cm diam Petri dish with fine mesh nylon attached to a 1 × 5 cm opening on the side. For predators that were *conditioned* (Table 2), the dish was set on the platform of the wind tunnel as soon as they attacked a weevil, with the open side of the dish facing the semiochemical source (frontalin or ipsenol), which was located less than 5 cm upwind. Each exposure lasted 1 min and was repeated three times, with a 3 min interval between each exposure. Other individual *T. dubius* were identically *exposed* to the pheromone component, but were not provided any weevil prey during the exposure period; they

Table 2 Summary of all treatments used in the wind tunnel experiment testing the effect of olfactory experience on *Thanasimus dubius* response toward three bark beetle pheromone components

Group	N[a]	Treatment[b]	Feeding status	Exposure	Test
1	19	Conditioned	2 weevils	Ipsenol	Ipsenol
2	18	Conditioned	2 weevils	Frontalin	Frontalin
3	18	Exposed	2 weevils	Ipsenol	Ipsenol
4	18	Exposed	2 weevils	Frontalin	Frontalin
5	17	Control starved	2 weevils	None	Ipsenol
6	18	Control starved	2 weevils	None	Frontalin
7	19	Control well-fed	5–10 weevils	None	Ipsenol
8	19	Control well-fed	5–10 weevils	None	Frontalin

[a] N=sample size for each group; a total of N=146 beetles were tested

[b] Each exposure was performed twice, the day before and 1 h before the test

were fed two weevils at least 2 h after the exposure to a prey pheromone component. Other individual *T. dubius* ("*control starved*") were fed with two weevils after having been starved but had never experienced an exposure to a prey pheromone component. Finally, we needed to ensure that starvation would not modify predator responsiveness to a particular prey pheromone; thus, we included naive groups of predators that were *well-fed* (*ad libitum*). All treatment groups (*control starved, control well-fed, exposed*, and *conditioned*) were tested for responses to ipsenol or frontalin, i.e., eight groups (Table 2).

Statistical Analyses The number of predators that were captured twice in Experiment 1 were analyzed by using an exact *G* test of independence as implemented in PROC FREQ (SAS Institute Inc., 2003), with the rows representing the pheromone component on which a predator was first captured and the columns the pheromone component for which the second capture occurred. The numbers of recaptured insects were low; thus, we pooled them across the two sites (Black River Falls and Dell Creek). The exact *G* test analyzed whether first and second choices to pheromone components were independent. If predators were specialists with a particular pheromone preference, then one would expect more captures on the diagonal and more likely a significant test result. Generalists, on the other hand, should show a more even pattern of recapture along all cells and a non-significant test result. Additionally, the mean number of predators caught the first time for each prey pheromone was analyzed by using a three-way ANOVA with site and pheromone treatment as fixed effects and block as a random effect (PROC MIXED, SAS Institute Inc., 2003).

For Experiment 2, we first compared the numbers of upwind flights (defined as individuals landing upwind *and* displaying at least one stand up antennal behavior) among different semiochemical treatments for each trial with an exact *G* test of independence that analyzed whether flying upwind was independent of the nature of the odor source. We also performed pairwise comparisons among treatments with exact *G* tests and applied a sequential Bonferroni correction to judge significance (α=0.05, Sokal and Rohlf, 1995; PROC FREQ and PROC MULTTEST, SAS Institute Inc., 2003). Then, numbers of predators that flew upwind twice were compared with an exact *G* test of independence to determine whether upwind flight during the first trial was independent from upwind flight during the second trial.

In Experiment 3, we tested 146 individual *T. dubius* during 6 d. Each day represents a block for the analysis. For each group shown in Table 3, N=17 to 19 predators were tested. We first used logistic regression to test whether the likelihood of flying upwind was influenced by the semiochemical or by experience. Our model used a binomial dependent variable Y that could take the values Y=0 or Y=1 (landing upwind or not). The two independent variables, the semiochemical and experience treatments, were both categorical. We also incorporated a block effect in this model by using PROC GLIMMIX (SAS Institute Inc., 2003). For each pheromone component, we also ran pairwise comparisons among experience treatments by using exact *G* tests and applied a sequential Bonferroni correction to judge significance (α=0.05, Sokal and Rohlf, 1995; PROC FREQ and PROC MULTTEST, SAS Institute Inc., 2003). We used this exact *G* test because the logistic regression analysis could not provide pairwise comparisons among the four experience treatments (*control starved, control well-fed, exposed*, and *conditioned*). These pairwise comparisons are still useful to rank the effect of a particular experience treatment relative to the attractiveness of the semiochemical. The mean rate of antennal behaviors/individual/min was square-root transformed and analyzed by using a three-way ANOVA with experience and semiochemical treatment as fixed effects and block as a random effect (PROC MIXED, SAS Institute Inc., 2003). To compare the effects of the experience treatment for each pheromone, we performed separately a two-way ANOVA with experience treatment as a fixed and block as a random effect (SAS Institute Inc., 2003, PROC MIXED). We also performed pairwise comparisons with the Tukey-Kramer multiple comparison procedure to judge significance (α=0.05, Sokal and Rohlf, 1995).

Results

Experiment 1: Mark-Release-Recapture and Field Choice Test A total of 327 *T. dubius* was caught in 2006 and 2007, and 9.2% of these were recaptured. There was an effect of site ($F_{1,5}$=8.97; P=0.03) on beetle catch, but the semiochemical treatment × site interaction was not significant ($F_{2,10}$=0.95; P=0.42), indicating there was no difference in

Table 3 Results of the mark-release-recapture experiment showing the number of *Thanasimus dubius* that were caught twice (N=30) in response to three bark beetle pheromone components at two sites in Wisconsin, 2006–2007

		Second capture		
		FR[a]	IS[a]	ID[a]
First capture	FR	4	1	5
	IS	3	1	5
	ID	4	1	6

Thanansimus dubius adults released following initial capture: N=327. Of these, 9.2% were recaptured

[a] *FR* frontalin, *IS* ipsenol, and *ID* ipsdienol

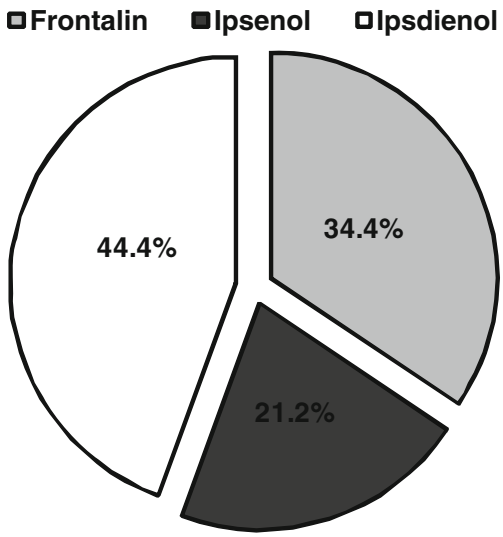


Fig. 2 Field responses of adult *Thanasimus dubius* in Wisconsin: Percentages of predators choosing frontalin, ipsenol, or ipsdienol during first capture (N=340)

preference between Dell Creek and Black River Falls. Overall, there was no difference in preference for prey pheromone components during first capture (Fig. 2, $F_{2,10}$= 3.45; P=0.07). The results (Table 3) show that the first and second choices were independent (exact G test, G=0.11, P=0.999), indicating that each individual *T. dubius* acted as generalist in its response to these prey pheromone components.

Experiment 2: Breadth of Kairomonal Range in Wild Thanasimus dubius We tested N=220 *T. dubius* adults (wild individuals from Wisconsin) in the wind tunnel (Fig. 3). During the first trial (Fig. 3**a**), the highest percentage of upwind flights was obtained when predators were exposed to sulcatol (73%), which was significantly higher compared to frontalin (35%) (exact G test, G=11.60; P=0.017 after Bonferroni correction). In the second trial (Fig. 3**b**), ipsenol, ipsdienol, and α-pinene were all more attractive than frontalin (exact G test, after Bonferroni correction, respectively: G=10.12; P=0.029; G=18.81; P<0.001; G= 13.20; P=0.007). The test of independence for insects responding twice was not significant (Table 4), suggesting that the response to a particular semiochemical during the first trial was independent from response on the second trial (exact G=5.09; P=0.997). As in Experiment 1, this result suggests that individual *T. dubius* act as generalists. In both the first and second trials, predators flew upwind more often when exposed to a semiochemical treatment relative to the no stimulus control (exact G test, first trial G=34.79; P<0.001 and second trial G=47.02; P<0.001); few insects (5%) responded to the no stimulus control.

Experiment 3: Effect of Experience on Thanasimus dubius Response: Upwind Flights Overall, experience greatly influenced the probability of upwind flight (logistic regression, χ^2=12.83; df=3; P=0.005; Fig. 4). The semiochemical treatment had no significant effect on upwind flight (logistic regression, χ^2=2.31; df=1; P=0.128), and the semiochemical × experience interaction was not significant (logistic regression, χ^2=1.93; df=3; P= 0.587). However, pairwise comparisons of the treatments within each semiochemical treatment suggested an effect of experience on the response to ipsenol (exact G test= 14.51; df=3; P=0.003), whereas the effect was not significant for frontalin (exact G test=4.52; df=3; P= 0.248). In particular, 63% of *T. dubius* that were *conditioned* flew upwind to ipsenol and this difference was significantly higher than either the control group *well-fed* or the control group *starved* (Fig. 4). Simple exposure also produced a moderate increase of upwind flights toward ipsenol (39%), but was not significantly different from the *control well-fed* or the *conditioned* groups.

Experiment 3: Effect of Experience on Thanasimus dubius Response: Antennal Behaviors The mean number of stand up antennal behaviors/individual/min to ipsenol was affected by experience (Fig. 5), similar to the trend observed for upwind flight (mixed model ANOVA; $F_{3,138}$=3.29; P= 0.023). *Conditioned* individuals were overall more responsive than *well-fed* ones (Tukey-Kramer, P=0.029). There

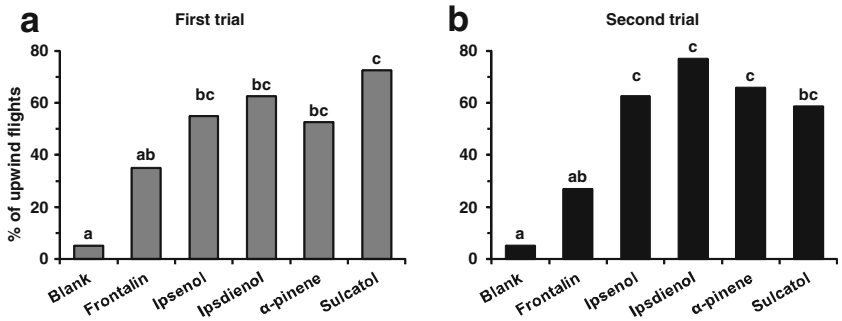


Fig. 3 Percentages of wild adult *Thanasimus dubius* flying upwind: **a** First trial, and **b** Second trial (N=220). Bars capped with different letters are significantly different (P<0.05)

Table 4 Number of field-collected *Thanasimus dubius* flying upwind in a wind tunnel in both of two trials to one of five semiochemicals

		Second trial				
		FR[a]	IS[a]	ID[a]	SU[a]	AP[a]
First trial	FR	2	3	2	3	3
	IS	1	4	1	2	4
	ID	2	5	5	2	4
	SU	2	3	4	3	4
	AP	2	3	5	2	3

N=74 on 200 individuals tested

[a] *FR* frontalin, *IS* ipsenol, *ID* ipsdienol, *SU* sulcatol and *AP* α-pinene

also was a highly significant effect of semiochemical on antennal response (mixed model ANOVA; $F_{3,138}$=14.84; P< 0.001), meaning that predators were more responsive to ipsenol than frontalin. The experience × semiochemical interaction was not significant (mixed model ANOVA; $F_{3,138}$=2.33; P=0.077). For ipsenol, the number of antennal behaviors was almost four times higher in *T. dubius conditioned* to ipsenol relative to those *starved* (Tukey-Kramer, P=0.001) (Fig. 5). For frontalin, there was no significant difference in the mean number of stand up antennal behaviors/individual/min (mixed model ANOVA; $F_{3,65.4}$=0.86; P=0.466) (Fig. 5).

Discussion

Our results suggest that *T. dubius* populations from Wisconsin consist of generalists in terms of response to kairomonal signals. Indeed, mark-release-recapture experiments showed that individual *T. dubius* are able to respond to all three major prey pheromone components that we deployed (frontalin, ipsenol, and ipsdienol). The wind tunnel experiment with adults collected in Wisconsin confirmed the breadth of *T. dubius* responses toward various semiochemicals, including another kairomone, sulcatol, and the tree volatile α-pinene. The individuals in the wind tunnel experiments initially were captured by using frontalin and turpentine, and, hypothetically, could represent a subset of the population that has a preference for these semiochemicals. However, it seems unlikely that these adults represented a subset of the population with a particular semiochemical preference because in the subsequent studies they responded to all semiochemicals (Table 2, Fig. 3). Furthermore, the mark-release-recapture results suggest that *T. dubius* initially captured on any pheromone were attracted to all others. Similar results were also obtained from individuals collected in Louisiana and southern Illinois (Costa and Reeve, unpublished data), suggesting that the present results are not restricted to *T. dubius* populations from Wisconsin.

The ability of adult *T. dubius* to respond to a variety of bark beetle semiochemicals is also a feature of other clerid predators that are distributed in the western U.S., such as *Enoclerus lecontei* (Wolcott) and *Enoclerus sphegeus* (Fabricius) (Seybold et al., 1992). This strategy may have evolved to enable *T. dubius* to locate sufficient prey resources despite drastic fluctuations in abundance (Turchin et al., 1991; Reeve and Turchin, 2002). One disadvantage of this strategy could be reduced efficiency in locating and utilizing any particular prey species, but it might be optimal when switching costs (e.g., longer prey handling time) are minimal (Stilmant et al., 2008).

The results of the present study also demonstrate that semiochemically naive predators became more attracted toward ipsenol after having experienced conditioning with this kairomone and a reward. Predator responses rose from 12% of upwind flights toward ipsenol for naive individuals to 63% for those conditioned. Trained *T. dubius* also performed significantly more stand up antennal behaviors than naive

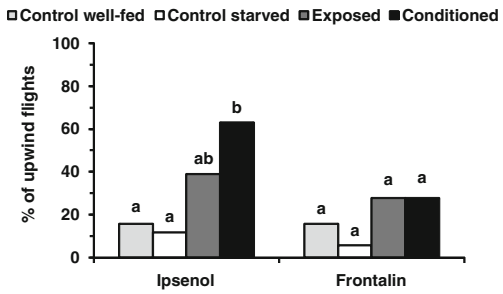


Fig. 4 Effect of previous olfactory experience on *Thanasimus dubius* response. Percentages of adult *T. dubius* flying upwind toward ipsenol or frontalin (N=146). For each prey pheromone component (ipsenol or frontalin), bars capped with different letters indicate a significant difference (P<0.05)

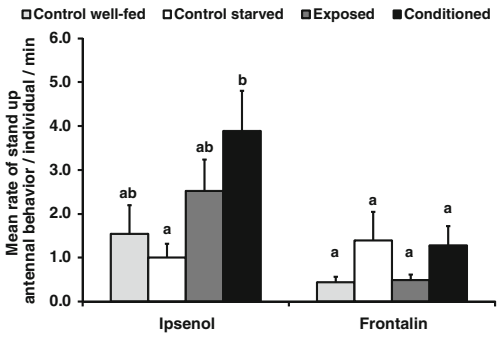


Fig. 5 Effect of previous olfactory experience on *Thanasimus dubius* response. Mean rate of stand up antennal behaviors (± S.E.) observed per individual in the wind tunnel (N=146). For each prey component (ipsenol or frontalin), bars capped with different letters indicate a significant difference (P<0.05)

ones. Control individuals that experienced a 10-d period of starvation were as responsive toward prey pheromones as were those that were well fed, thus showing that the increase of attraction toward ipsenol seen in experienced predators was not due to starvation. Combining our results with previous work on the carabid beetle, *Pterostichus melanarius* (Illiger) (Plotkin, 1979), or on coccinellids (e.g., De Jean et al., 2003) suggests that olfactory experience can influence the behavior of individuals in several groups of predatory beetles.

We showed here an effect of experience on predator response toward ipsenol because conditioned predators were more responsive than naive ones. However, we cannot exclude that a sensitization component may have acted as well in the significant increase of attractiveness shown in conditioned *T. dubius* (63%), because it was not significantly different from the exposed ones (39%). Thus, the present results do not allow us to distinguish whether sensitization, associative learning, or both are involved in this pattern. Yet our results provide a possible explanation for the pattern observed by Reeve et al. (2009) where the relative response to ipsenol increased in areas where *I. grandicollis* (an ipsenol emitter) was present, but *D. frontalis* (a frontalin-emitter) was absent or at a low population density.

Attraction toward frontalin was slightly increased in exposed and conditioned predators (28%), especially in comparison to ipsenol. Training for a response to frontalin may possibly require more exposure to significantly increase the preference. At this release rate (0.5–1 mg/d), naive predators were more responsive to ipsenol than to frontalin in the wind tunnel, and possibly a higher release rate for frontalin would elicit a stronger response. Additionally, these results suggest that *T. dubius* response to this kairomone could be a fixed trait because the sensitization and learning treatments had no effect on the response. This is in agreement with field-trapping studies showing that this predator invariably displays a strong response to frontalin, even in areas where frontalin-emitting prey appear to be at low population densities (Billings and Cameron, 1984; Aukema and Raffa, 2005; Reeve et al., 2009). The relatively low response of adult *T. dubius* to frontalin in the wind tunnel was somewhat surprising and, at first, seems to disagree with the results of the mark-release-recapture experiment. However, we did not include any visual silhouettes in the wind tunnel while testing the semiochemicals. This was not the case in field experiments where the black silhouette could enhance the response to frontalin. Indeed, adult *T. dubius* have been shown to use visual cues in concert with frontalin in the field (Strom et al., 1999; Reeve et al., 2009).

How *T. dubius* populations cope with unpredictable and patchily distributed prey resources in the field has not been fully elucidated. Individual predators in this study seem to act as generalists based on odor preference for prey. The ability of individuals to respond to various kairomones is likely to help *T. dubius* populations to cope with drastic changes in bark beetle abundance. Our results also suggest that previous olfactory experience plays a role in the response of *T. dubius* to certain prey pheromones (ipsenol) but not others (frontalin). Further research on learning could use naive *T. dubius* conditioned with an artificial flavor such as vanilla (Lewis and Tumlinson, 1988), which is not innately attractive (Costa and Reeve, 2011), to determine whether associate learning, sensitization, or both are involved.

Finally, we hope that future studies will investigate whether experience generally affects olfactory behavior in other clerid species (e.g., *Enoclerus* spp.) that attack bark beetles and are generalists in terms of diet. Additional studies could focus on clerids that also are predatory but whose prey inhabit herbaceous plants (such as *Phyllobaenus* or *Trichodes* spp.) and, thus, deal with different resources (Foster, 1973; Opitz, 2002). Given that many of these predators also experience large changes in their prey communities, the learning of prey kairomones may be a common strategy within this taxonomic group.

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(southwestern USA), *P. ponderosa* exhibits substantial polymorphism in the constitutive monoterpene composition of its oleoresin (Smith et al., 1969; Latta et al., 2003; Davis and Hofstetter, 2011). This polymorphism can occur at highly localized scales, and multiple chemical variants of *P. ponderosa* often are interspersed geographically (Latta et al., 2003). In addition to inter-tree polymorphism, the monoterpene composition of conifer oleoresin can vary within an individual (Nerg et al., 1994; Barnola et al., 1997). For example, the monoterpene composition of *P. ponderosa* needles often is different from the monoterpene composition of phloem or xylem oleoresins, which may also differ (Latta et al., 2000). In other conifer systems, enantiomeric compositions and relative amounts of monoterpenes also can vary within individual trees (Persson et al., 1996). However, relative monoterpene composition of conifer phloem oleoresin is a quantitative trait and under moderate to strong genetic control (Hanover, 1971, 1992).

It has been long believed that the chemical composition of tree resin can mediate bark beetle population dynamics (Hopkins, 1916; Raffa et al., 2005). There are few experimental demonstrations that oviposition behavior or fecundity of *Dendroctonus* spp. bark beetles is regulated by intraspecific variation in the monoterpene composition of host conifers (Sturgeon, 1979; Wallin and Raffa, 2004). Our objective was to assess the effect of two common monoterpene compositions (henceforth, 'chemotypes') of *P. ponderosa* in northern Arizona in combination with the chemical history of *D. brevicomis* on the rate of host acceptance, egg gallery construction behavior, and fecundity. We tested two hypotheses: (1) beetle performance (i.e., host acceptance, egg gallery construction, and fecundity) varies between host tree chemotypes, and (2) beetle performance is mediated by insect chemical history. We addressed these hypotheses by using a series of rearing assays in which we controlled for the chemotype of host material in which the beetles developed. By evaluating the F_1 generation, we were able to compare beetle performance across chemotypes, and between natal and non-natal chemotypes. Experimental evidence suggests that both oviposition behavior and fecundity were mediated by beetle chemical history, but performances were equivalent between tree chemotypes.

Methods and Materials

Oleoresin Collection and Compound Identification To collect resin from *Pinus ponderosa* var *scopulorum* trees, a No. 16 resin collection punch (C.S. Osborne Co., Harrison, NJ, USA) was used, and a 25 ml glass collection vial was inserted into the resulting hole. Holes for oleoresin collection were punched through the outer bark at a height of 1.47 m, and approximately 3 mm into the phloem layer. This sampling height and depth is relevant to *D. brevicomis*, as the beetle primarily attacks stems at a height range of 0.5–10 m, and generally only colonizes the phloem layer (Wood, 1982). Since we only wished to analyze oleoresin collected from the phloem, we were careful to ensure that oleoresin collection holes did not puncture the xylem. After 24 h (Latta et al., 2000), vials were collected, and this typically yielded 10–25 g of resin for each vial. This time period reflects a collection of constitutive (vs. induced) resin (e.g., Wallin et al., 2003), and previous studies have shown that mechanical stem wounding has little effect on monoterpene cyclase activity in phloem tissues of *P. ponderosa* (Lewisohn et al., 1991). Oleoresin samples from tree populations in northern Arizona were collected between 15 May and 15 July 2008, and stored at −20°C until used in gas chromatography. A sample of constitutive phloem oleoresin was collected from every ponderosa pine (N=194) over a 2 ha area, at an elevational range of 2100–2200 m (Davis and Hofstetter, 2011).

To prepare oleoresin samples for analysis, 1 g of sample from each tree was dissolved in hexane (GC grade, > 99.0%) to a total volume of 2.0 ml. Fifty μl of this solution were further diluted to 5 ml with GC grade hexane, and 50 μl of 9.48 mg/ml L-fenchone (>98.0% chemical purity, 99.5% isomeric purity, Aldrich, Milwaukee, WI, USA) were added as an internal standard. The same concentration of fenchone was added to calibration standards. Six calibration standards, each with increasing concentrations of monoterpenes, were prepared along with two check standards. All standards were created from a stock solution. The stock solution contained high purity monoterpenes (Table 1) dissolved in GC grade hexane. The monoterpenes α-pinene, β-pinene, myrcene, Δ-3-carene, limonene, and terpinolene were tested. All samples and standards were transferred to 2 ml glass vials with ivory PTFE/red rubber septa. Vials were stored in a freezer at −20°C until analysis.

A Hewlett-Packard 5890 Series II Gas Chromatograph (GC) (Agilent, Santa Clara, CA, USA) with a 6890 Series auto-sampler injector and a 100 vial tray were used for the analysis of the oleoresin samples. The system used on-column injection and a flame ionization detector (FID), and a J&W Scientific (Agilent, Santa Clara, CA USA) capillary GC DB-1 column (30 m×0.25 mm i.d.; film thickness of 0.25 μm). The column was heated to 300°C for 1 h prior to the start of analysis to condition the column and remove any residual compounds from previous analyses. Carrier gas (He) flow rate was 20 ml/min; makeup gas (N_2) flow rate was 10 ml/min; and detector gas (H_2 and compressed air) flow rates were 30 ml/min and 370 ml/min, respectively. Injector temperature was 275°C, and the detector temperature was 300°C. Injection mode was manual, and the temperature program

Table 1 The mean concentrations of six monoterpenes (± s.e.) collected from phloem oleoresins in two chemotypes of ponderosa pine, *Pinus ponderosa* var *scopulorum*, found in northern Arizona (southwestern USA.)[a,b,c]

Chemotype	Monoterpene concentration (mg/g resin)					
	α-Pinene	β-Pinene	Myrcene	Δ-3-Carene	Limonene	Terpinolene
α-Pinene	169.75±35.76	8.05±2.98	14.06±1.74	5.78±2.78	32.08±3.83	0.78±0.23
Δ-3-Carene	71.48±1.76	5.19±1.35	10.38±0.75	113.57±0.57	13.14±1.50	7.31±0.17

[a] From the oleoresin samples of 194 ponderosa pines, 24.7% were the α-pinene chemotype (N=48) and 37.1% were the Δ-3-carene (N=72) chemotype. These chemotypes are geographically intermixed. All samples were collected in Coconino County, Arizona

[b] Chemical purities, (enantiomeric purities), and commercial sources of calibration standards used in GC analysis are as follows: (1*R*)-(+)-α-pinene 99.0% (96.5%), Sigma-Aldrich, St. Louis, MO, USA; (1*S*)-(−)-β-pinene 99% (98.5%), Aldrich Chemical Company, Milwaukee, WI, USA; myrcene (technical) >75% (achiral), Aldrich Chemical Company, Milwaukee, WI, USA; Δ-3-carene 95.7% (95%), Aldrich Chemical Company, Milwaukee, WI, USA; (*R*)-(+)-limonene 97% (97.5%), Aldrich Chemical Company, Milwaukee, WI, USA; terpinolene 85% (achiral), TCI America, Portland, OR, USA

[c] Chemotype data modified from Davis and Hofstetter, 2011

was as follows: Start 45°C, hold 0.1 min; ramp 25°C/min to 70°C, hold 0.1 min; ramp 2°C/min to 96°C, hold 0.5 min, ramp 40°C/min to 300°C, hold 1 min.

A SRI Instruments Peak Simple Model 202 data system and software were used to acquire the FID signal from the GC and draw chromatograms. Monoterpenes were identified by retention time. This software identifies each peak within a defined window of expected retention time. Calibration curves were created for each monoterpene by plotting the corrected peak area (area of the monoterpene peak divided by the area of the fenchone peak for that sample) vs. the known monoterpene concentration for each calibration standard. Calibration curves were used to determine the monoterpene concentration in each diluted sample. A duplicate and a check standard were analyzed every 10 samples, and all results were found to be repeatable. Results were reported as mg of each monoterpene per g of oleoresin sample.

Bark Beetle Collection *Dendroctonus brevicomis* were collected from Lindgren funnel traps [Lindgren, 1983; PheroTech Inc. (now Contech Enterprises, Inc.), Delta, BC, Canada] baited with *D. brevicomis* enhanced pheromone lures consisting of *exo*-brevicomin, frontalin, and α-pinene (Synergy Semiochemicals Corp, Burnaby, BC, Canada, P130 Lot No. WPP10416). The enantiomeric composition, chemical purity, and release rate of each compound in the trap baits were as follows: *exo*-brevicomin 50%-(−), 99%, 1.5 mg ±0.2 mg/d; frontalin 50%-(−), 99%, 1.5 mg±0.2 mg/d; and α-pinene 80%-(+), 99%, 2.5 mg±0.3 mg/d. Traps were placed in forested areas predominated by *P. ponderosa* in northern Arizona at elevations of 2100–2200 m. Beetles were collected live and placed individually into clear, size 0 gelatin capsules (Torpac, Fairfield, NJ, USA, Lot No. 1100049271). Beetles were stored in the lab in dark environmental chambers at 5°C until introduced into host material. Beetles were held in chambers no longer than 24 h prior to use. Beetles were collected from 1 May 2009 to 1 October 2009 and from 1 May 2010 to 1 October 2010.

Rearing Experiments Four chemotypes were identified based on our surveys of monoterpene composition (consistent with Latta et al., 2003; result described in Davis and Hofstetter, 2011). For the purposes of the present study, only two host chemotypes were used: 1) Constitutive oleoresin composed primarily of α-pinene; or 2) Constitutive oleoresin composed primarily of Δ-3-carene. The α-pinene chemotype is characterized by high concentrations of α-pinene and limonene, and very low concentrations of Δ-3-carene and terpinolene. Conversely, the Δ-3-carene chemotype exhibits high concentrations of Δ-3-carene and terpinolene, low concentrations of limonene, and a 58% reduction in α-pinene relative to the previous chemotype (Table 1). The suitability of trees for use in rearing experiments was determined by chemotype and size [diameter at breast height (1.47 m) range 25–35 cm, height range 10–17 m]. Trees were felled and then cut into logs that were 30 cm in length. The ends of all logs were sealed with melted paraffin wax to prevent desiccation. For each series of rearing assays, host material of each chemotype was collected from the same location and on the same day. In 2009, six trees of each chemotype (N=12 total) were felled and used, and in 2010, seven trees of each chemotype (N=14 total) were felled and used. Each tree typically resulted in approximately 25 logs, and the use of log material in rearing experiments was randomized.

The replication of experimental units was performed at the log level, as described previously (Davis and Hofstetter, 2009). Beetles were introduced into logs at a density of 1 pair/dm^2. This resulted in a mean introduction rate of approximately 20 (mean = 19.78) females per log. Holes were drilled with a bore diameter of 3 mm into logs, to the

phloem layer. A gelatin capsule containing a female *D. brevicomis* was placed inside the resulting hole. All introductions were performed simultaneously for each log. After 48 h, each log was examined to ensure that females had successfully initiated tunneling. Females that did not begin tunneling after 48 h were removed from logs and discarded. For each successful female, a male was introduced into the hole, as described above. Each log was placed into a metal rearing canister. In 2009, 1,694 trap-caught beetles were introduced into 46 logs (23 α-pinene chemotype; 23 Δ-3-carene chemotype), and in 2010, 2,666 trap-caught beetles were introduced into 62 logs (32 α-pinene chemotype; 30 Δ-3-carene chemotype).

As adults from the F_1 generation emerged (42–50 d following initial introductions), they were placed into gelatin capsules and stored in the lab in dark environmental chambers at 5°C until used. We discerned re-emerging adults from offspring based on the length of time it took for beetles to appear in rearing canisters following introduction. Re-emerging adults typically appeared in rearing canisters after approximately 10–20 d, whereas, as noted above, F_1 offspring typically appeared 42–50 d following introductions. F_1 beetles were held for 48–72 h prior to use in experiments (Paine and Birch, 1983). In 2009, 1,755 F_1 beetles were collected, and in 2010 2,903 F_1 beetles were collected. The chemotype of the host material that each beetle was collected from was recorded. F_1 offspring beetles were introduced into host material that was the same chemotype as the log they developed in (natal) or the opposing chemotype (non-natal), and female acceptance rate was assessed at 48 h. In 2009, 1,610 F_1 beetles were introduced into 35 logs (13 α-pinene chemotype; 22 Δ-3-carene chemotype), and in 2010, 1,316 F_1 beetles were introduced into 34 logs (18 α-pinene chemotype; 16 Δ-3-carene chemotype).

Adults from the F_2 generation were collected and tallied but not used further in experiments. In 2009, 2,143 F_2 beetles were collected, and in 2010, 1,750 F_2 beetles were collected. Following complete emergence of all beetles from each log, the outer bark was removed and the length of each egg gallery was measured. Thus, for each log, the proportion (%) of females that entered the log, the mean egg gallery length (cm), and the mean number of offspring that were produced by each female were determined.

Statistical Analyses All statistical tests incorporated a type I error rate of $\alpha=0.05$, and all statistics were computed by using the software JMP 8.0 (SAS Institute). The rearing experiment was analyzed as a two-way ANOVA. Our model accounted for chemotype of host material ($N=2$ α-pinene chemotype, Δ-3-carene chemotype) and chemical history ($N=3$: unknown/trap-caught, natal, non-natal) of beetles. These were treated as fixed effects on the response variables of proportion (%) female acceptance, mean egg gallery length, and mean number of offspring per female. The interaction of chemotype X chemical history also was evaluated. When significant differences were detected in the model, Tukey's HSD test was used to separate means. In addition, the correlation between mean egg gallery length and mean number of offspring per female was analyzed by using least-squares linear regression.

Results

The proportion of female beetles that accepted host material did not vary with chemotype ($F_{1,\ 175}=1.29$; $P=0.256$), beetle chemical history ($F_{2,\ 175}=1.03$; $P=0.357$), or the chemotype X chemical history interaction ($F_{2,\ 175}=2.21$; $P=0.112$). The mean rate of host acceptance was 74.3% for female beetles that developed in the α-pinene chemotype and 79.4% for female beetles that developed in the Δ-3-carene chemotype. The mean rate of host acceptance was 73.2% for trap-caught female beetles; 79.6% for female beetles that were introduced to natal host material; and 77.7% for female beetles that were introduced to non-natal host material.

Mean egg gallery lengths did not vary between host chemotypes ($F_{1,\ 175}=0.00$; $P=0.962$), but mean egg gallery lengths varied with beetle chemical history ($F_{2,\ 175}=9.22$; $P<0.001$). Mean egg gallery length was 25.11 cm (± 0.70 s.e.) for trap-caught beetles; 23.75 cm (± 1.25 s.e.) for beetles in natal host material; and 18.70 cm (± 1.30 s.e.) for beetles in non-natal host material. Parent beetles produced oviposition galleries that were 24.3% longer on average when reared in host material that was chemically similar to their natal host (Fig. 1). Mean egg gallery length and the mean number of offspring produced per female were weakly correlated ($r^2=0.03$; $F_{1,\ 175}=5.67$; $P=0.018$; equation: [Offspring per female] = 0.829+0.077 * [mean gallery length (cm)]). Mean egg gallery lengths did not vary by the chemotype X chemical history interaction ($F_{2,\ 175}=0.26$; $P=0.769$).

Mean number of offspring produced per female did not vary between host chemotypes ($F_{1,\ 171}=0.05$; $P=0.807$), although in non-natal hosts beetles reared in α-pinene host material produced more offspring than beetles reared in Δ-3-carene host material. Mean offspring production per female varied by beetle chemical history ($F_{2,\ 171}=3.58$; $P=0.029$). Trap-caught female beetles produced 3.10 (± 0.31 s.e.) offspring on average; beetles that developed in natal host material produced 2.52 (± 0.56 s.e.) offspring on average; and beetles that developed in non-natal host material produced 1.33 (± 0.57 s.e.) offspring on average. Beetles that developed in natal host material produced 52.8% more offspring, on average (Fig. 2), than those that developed in non-natal host

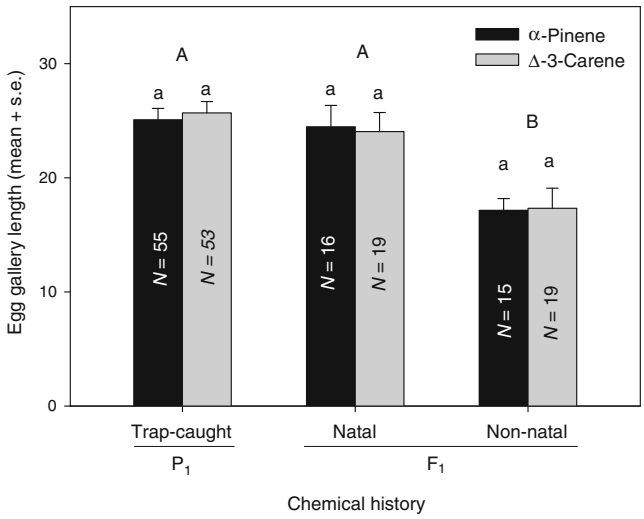


Fig. 1 The effect of host chemotype (black bars: α-pinene; grey bars: Δ-3-carene) and beetle chemical history on mean egg gallery length of *Dendroctonus brevicomis*. Error bars represent one standard error of the mean. The number of experimental units used to calculate each mean is shown for each bar. Lower case letters denote a separation of means test by chemotypes, and upper case letters denote a separation of means by beetle chemical history. P_1 indicates the parent generation, and F_1 indicates the reared generation

material. The chemotype X chemical history interaction was marginally non-significant ($F_{2,\ 171}$=2.45; P=0.088) on the response variable of mean offspring production.

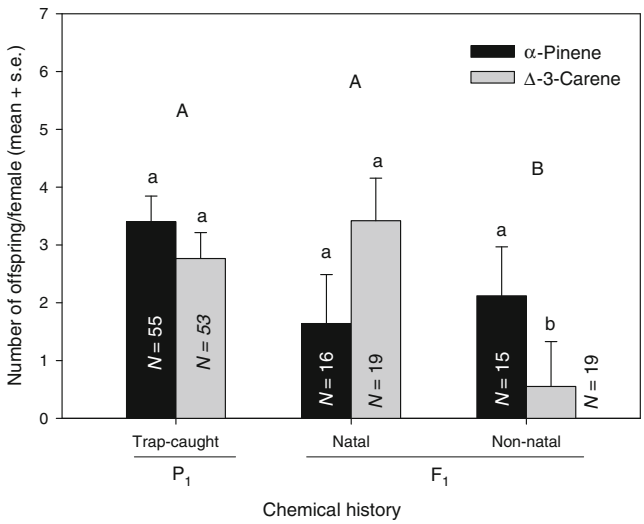


Fig. 2 The effect of host chemotype (black bars: α-pinene; grey bars: Δ-3-carene) and beetle chemical history on mean offspring production from *Dendroctonus brevicomis* pairs. Error bars represent one standard error of the mean. The numbers of experimental units used to calculate each mean are shown for each bar. Lower case letters denote a separation of means test by chemotypes, and upper case letters denote a separation of means by beetle chemical history. P_1 indicates the parent generation, and F_1 indicates the reared generation

Discussion

Our experiments support the hypothesis that *D. brevicomis* performance is mediated by chemical history, but we did not find evidence to support the hypothesis that performance varies between two host *P. ponderosa* chemotypes in Arizona. We show that egg gallery construction behavior and fecundity were both influenced by insect chemical history. In both cases, F_1 beetle performance was superior when beetles developed in host material that was chemically similar to their natal host. Interestingly, rates of host acceptance did not appear to vary across host chemotype or by beetle chemical history. Thus, although we do not show evidence that host acceptance, egg gallery construction, or fecundity are related to a particular chemotype, we did find evidence to suggest that some aspects of *D. brevicomis* performance are mediated by the monoterpene composition of its previous host. Our results are consistent with the findings of Davis and Hofstetter (2011), which showed that chemical legacy impact the growth of nutritional fungal symbionts associated with *D. brevicomis*.

The results of our experiment warrant discussion of Hopkins' host selection principle (HHSP). Hopkins (1916, 1917) postulated that "insects become conditioned to the host on which they develop, and this will influence the adult female to deposit her eggs on the same species of host and might restrict her progeny to that host" (Walsh, 1864; Barron, 2001)." We assessed the effects of beetle chemical history both from a behavioral (i.e., host acceptance, egg gallery construction) and ecological (i.e., reproductive rate) perspective. Under the proposal that any mechanism causing larval host plant experience to affect oviposition decisions satisfies HHSP (Janz et al., 2009), our experiments provide evidence that both refutes and supports Hopkins' theory. Consistent with the results of Janz et al. (2009), we found no evidence that *D. brevicomis* was more likely to accept host material that was chemically similar to their natal hosts, which implies that restriction to a single chemotype in nature is improbable. However, we did find that *D. brevicomis* produced longer egg galleries in natal chemotypes, which suggests that some behavioral differences may exist among beetles that develop in differing chemotypes. Also, we showed that fecundity was higher on natal chemotypes. This implies that host plant chemistry may act rapidly as an agent of natural selection on *D. brevicomis* to produce lineages within endemic beetle populations that are adapted to a diversity of tree chemotypes. So, although we refute the notion of beetles becoming 'conditioned' to a particular chemotype (Corbet, 1985), we do find that there are likely to be consequences to beetle reproductive rates that result from the interaction between *D. brevicomis* and pine chemotypes (Futuyma and Peterson, 1985). Natural selection to this end is consistent with a genetic mechanism for HHSP, as

described by Barron (2001); and indicates that HHSP could perhaps benefit from further examination of oviposition behaviors within individual host plant species.

Several limitations of the experimental design should be noted. One shortcoming of our experiment is that a no-choice assay of host acceptance does not reflect a true estimation of host selection. In a natural setting, host selection trends may depart from the trends in acceptance that we observed due to fluctuations in temperature and precipitation, the presence of natural enemies, and stand or site conditions. Furthermore, we altered the influences of pheromonal communication and aggregation among beetles by deliberately introducing F_1 beetles to host material, and our experiment restricted F_1 beetles from flight. This could dramatically affect the likelihood that beetles will accept a host (Byers and Wood, 1981; Byers et al., 1984). Additionally, our trapping method for parent beetles may have selected for a portion of the beetle population that responded to the specific semiochemicals that we used for trapping (*exo*-brevicomin, frontalin, and α-pinene). Thus, it is possible that another portion of the population may have responded to other enantiomers of bicyclic acetals and to other monoterpene co-attractants, and that these insects may have produced offspring that performed differently.

Primary chemistry (e.g., nitrogen, carbohydrates, phosphorous) and other secondary chemicals (e.g., phenols, calcium oxalate) present in host material may have affected the responses tested in this experiment. However, we attempted to control for these factors by randomizing our use of host material across 26 trees. In addition, initial chemotyping was performed on standing trees, rather than logs. Monoterpenes rapidly volatilize, and sectioning trees into logs may have elicited an induced response from host material, so it is possible that monoterpene compositions differed over time in log material. Thus, in the context of this experiment the only factor that we can attribute differences in *D. brevicomis* oviposition behavior and fecundity to is the initial chemical factor that we controlled for: i.e., constitutive monoterpene composition of phloem oleoresins of living trees. However, it should be recognized that other chemical factors could have contributed to the treatment effects observed here.

Despite these limitations, our introduction technique achieved an average acceptance rate of 76.8%, so a majority of beetles entered host material despite the absence of conspecific pheromones, alterations to tree material, and the restriction of flight. We noted that fecundity is reduced when *D. brevicomis* are reared in a laboratory setting (approximately 3 offspring/female), compared to approximately 10 offspring per female in study trees in the field (Davis and Hofstetter, 2009). Collectively, a disturbance to pheromonal communication, restriction of flight, our trapping method, and the reduction of fecundity may contribute to different behaviors between laboratory-reared and natural populations of *D. brevicomis*. These factors should be taken into consideration when comparing the results of our experiment to beetle behaviors in Nature.

In summary, we found support for the hypothesis that insect chemical history can mediate egg gallery construction and fecundity of *D. brevicomis*. This result may be consistent with a genetic mechanism for HHSP (Barron, 2001). We showed here that the monoterpene composition of natal hosts is likely to influence beetle performance on subsequent hosts. However, we did not find any support for the hypothesis that beetle oviposition behavior or fecundity varies fundamentally between chemotypes of *P. ponderosa*. Rather, our experiment suggests that monoterpene composition of *P. ponderosa* may act rapidly as an agent of selection on *D. brevicomis* populations, and that multiple lineages adapted to a variety of pine chemotypes may exist in natural populations. This could indicate that *D. brevicomis* population dynamics are regulated by the chemical diversity present in populations of their host pine. For example, high-density beetle populations (outbreaks) are rarely observed in northern Arizona (USDA Forest Service, 2010), where pine chemical diversity is high (Latta et al., 2003). In contrast, outbreaks occur at relatively frequent intervals in northern California, where host pine chemical diversity is comparatively low (Sturgeon, 1979). We detected substantial chemical diversity in *P. ponderosa* at a scale of 2 ha, and found that beetle oviposition behaviors and fecundity vary across the chemotypic diversity at this scale. Future work in this system should focus on assaying host selection by beetles with controlled chemical histories in a field setting.

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and Tueller, 2001; Santoro et al., 2001; McHugh et al., 2003; Sullivan et al., 2003; Wallin et al., 2003; Elkin and Reid, 2004; Hood and Bentz, 2007; Six and Skov, 2009). Lombardero et al. (2006) found *Ips pini* (Say) and *Ips grandicollis* (Eichhoff) preferred fire-injured *Pinus resinosa* Aiton, suggesting such behavioral adaptations may underlie a significant ecological relationship between some bark beetles and their hosts. From a population dynamics perspective, fire-injury may provide a refuge for some bark beetles during their endemic phase, but not by itself necessarily lead to outbreaks (Powell et al., 2012).

This high variability poses substantial challenges to natural resource managers, who must make important and often contentious policy decisions involving fire and insect suppression, controlled burns, fuel accumulation to restore more natural conditions, and protection of human habitations. Understanding more about the mechanisms by which fire injury affects tree susceptibility to bark beetles could potentially address this problem.

Conifers are defended against bark beetle attack through several integrated mechanisms. Constitutive defenses act as a first line of defense and include physical barriers such as thick bark and lignin (Franceschi et al., 2005) and chemical barriers such as terpenoids and phenolics (Martin et al., 2003). Inducible defenses are triggered by insect or pathogen invasion and include formation of necrotic tissue that confines the invader (Paine and Stephen, 1988) and increased terpene and phenolic production, which deters establishment (Cook and Hain, 1988; Raffa and Smalley, 1995; Franceschi et al., 2005). In particular, increased monoterpene concentrations, and sometimes changes in their relative proportions, have deterrent, adulticidal, and ovicidal activities against bark beetles, and inhibit growth and germination of their fungal symbionts (Coyne and Lott, 1976; Raffa et al., 2005; Keeling and Bohlmann, 2006; Seybold et al., 2006; Bonello et al., 2006; Erbilgin et al., 2007; Bohlmann and Gershenzon, 2009; Zulak and Bohlmann, 2010). The ability to undergo induced total monoterpene accumulation appears particularly well correlated with tree survival in the field (Raffa and Berryman, 1982; Boone et al., 2011).

Stressors, such as severe drought, defoliation, lightning, and root disease, can reduce tree defenses against bark beetles (Christiansen et al., 1987; Flamm et al., 1993; Klepzig et al., 1996; Lombardero et al., 2006; Wallin and Raffa, 2001; Lewis and Lindgren, 2002; Turtola et al., 2003; Raffa et al., 2005). The effects of stress caused by fire injury on tree defenses, and its subsequent effect on bark beetle colonization and reproductive success, however, are less well known. Defensive resin flow has been shown to increase with fire injury in some conifers, but only several months post-fire (Wallin et al., 2003; Lombardero et al., 2006; Knebel and Wentworth, 2007). Our knowledge of how fire affects host chemical concentration and composition is even more limited. In one study, dead, fire-injured lodgepole pine (*Pinus contorta* var. *latifolia* Douglas) (Jakubas et al., 1994) had lower total volatiles than live uninjured trees. In another, total monoterpenes decreased 1 month post-fire in red juniper (*Juniperus virginiana* Lawson) (Campbell and Taylor, 2007). The defenses of fire-injured lodgepole pines that remain alive and hence potentially, susceptible to bark beetle attack, have not been examined, nor have the inducible chemical defenses that follow fire injury in any system.

The mountain pine beetle (*Dendroctonus ponderosae* Hopkins) is of major concern throughout the western United States and Canada. It recently has caused extensive mortality in both pine forests where it historically has undergone intermittent outbreaks and at higher latitudes and elevations than were previously common or recorded, an expansion attributable to changing climate (Kurz et al., 2008; Raffa et al., 2008; Safranyik et al., 2010). The primary host of the mountain pine beetle is lodgepole pine. During colonization attempts, it appears to benefit from its fungal symbiont, *Grosmannia clavigera*, in degrading lodgepole pine defenses (DiGuistini et al., 2011). The mountain pine beetle also exploits some host monoterpenes as precursors, stimulants, and synergists of the aggregation pheromones that it uses to coordinate the mass attacks need to overwhelm tree resistance (Wood, 1982; Blomquist et al., 2010).

We examined the effects of fire injury on lodgepole pine chemical defenses. Specifically, we tested whether fire affects the constitutive and induced total volatile concentrations, and their composition, in lodgepole pine phloem. We also tested whether mountain pine beetles caged onto trees with varying levels of fire injury would enter at similar frequencies.

Methods and Materials

Plot Description and Classification of Fire Injury Category The LeHardy fire began on July 30, 2008, and burned 3,777 ha of lodgepole pine and mixed lodgepole pine and spruce forest in Yellowstone National Park, U.S. A. Sampling took place 1 year post fire, in July of 2009. Trees in nine, 1 ha plots separated by at least 100 m within the burn were classified for degree of fire injury using the methods of Ryan (1982) and Powell et al., (2012). Measurements of percent basal, bole, and canopy injury, and cambium kill rating (ckr) were used to designate the injury to each tree as unburned, low, moderate, and high as follows: Unburned trees were healthy, and without fire injury. Low-injured trees had 0–10% injury on the tree base and bole, and a ckr of zero or one. Moderately-injured trees

had 11–49% injury on the tree base and bole, 0–10% injury of the canopy, and a ckr of two or three. Highly-injured trees had 50–100% injury on the tree base and bole, 10–100% injury of the canopy, and a ckr of three or four. Trees were at least 15 cm diam at 1.3 m.

Measurements were taken for additional variables that potentially could affect tree defenses. Tree-level variables included diameter at 1.3 m, tree height, presence and rating of dwarf mistletoe (*Arceuthobium americanum* Nuttall Ex. Engelmann) by using the method of Hawksworth (1979), and presence of *Cronartium* spp. stem rust by using the method of Geils and Jacobi (1984). We deliberately selected healthy trees without attacks by stem-boring insects; in a few cases, we found evidence of old attacks or new attempts between initial designation and final sampling, so this was also built into our model. Stand level variables included aspect, slope, and elevation.

Effects of Fire Injury on Constitutive and Induced Volatile Compounds Two pieces of phloem (approximately 5 cm long by 2 cm wide) were removed with a razor blade from 50 lodgepole pines from each fire injury category, sealed in plastic tubes, transported from the field in dry ice-cooled Styrofoam coolers, and frozen at −20°C until analysis. One phloem piece each was removed from the north and south aspects of the tree, or if the tree was moderately- or highly-fire-injured, they were removed from the burned or unburned portions of the tree. These served as the constitutive samples.

We elicited induced responses on the same trees by challenge inoculations that mimicked the combined entry of the mountain pine beetle and its major fungal symbiont, *Grosmannia clavigera*. We isolated *G. clavigera* by rolling live beetle larvae from lodgepole pine phloem onto malt extract agar, isolating and culturing at 25°C for 7 d by using the method of Wallin and Raffa (1999). A pure culture of *G. clavigera* was isolated and identified by its morphology by using microscopy and descriptions from Upadhyay (1981). Simulated attacks of 1 cm agar-fungi plugs were administered at the xylem/phloem interface by using sterile techniques. The 2 cm bark-phloem plug was replaced immediately to the site of introduction. Two inoculations were administered per tree, on the north and south aspects, or the burned or unburned portions for moderately- and highly-injured trees. We simulated attack at the same time when constitutive phloem was sampled. We sampled the reaction zone 7 d after simulated attack and stored the samples as above.

Compounds were extracted from finely chopped constitutive and induced phloem samples after 24 hr of agitation in 1 ml of hexane as described in Raffa and Smalley (1995). This solution was filtered with glass wool. The sample vials were rinsed twice with 250 μl of hexane, which resulted in a final volume of 1.5 ml volatile-hexane solution. One μl of 0.1% isobutylbenzene was added to each sample as an internal standard. This internal standard has been commonly used in previous, including recent, analyses of these compounds (Robert et al., 2010; Boone et al., 2011), thereby facilitating between-study comparisons. However, some variation could arise from different response factors to any common internal standard (Raffa and Steffeck, 1988), and likely increases when non-monoterpene hydrocarbons are included. The remaining phloem was dried at 25°C for 1 wk and weighed.

Samples were analyzed with a Shimadzu 17 gas chromatograph with an Agilent Technologies cyclodex chiral column: 30 m long, 0.25 mm internal diam, and 0.20 μm film thickness with Helium as the carrier gas. Each analysis began at an initial temperature of 60°C for 10 min, followed by an increase in 5°C per min until 160°C. The total run for each sample lasted 69 min. Thirty pure volatile standards from Sigma-Aldrich for those commonly found in pines also were run using the above described method. We compared the retention times of these standards with the peaks on the chromatogram produced after the run of each sample. Milligrams of each compound were calculated by integrating its area under the curve and the internal standard, and we multiplied this value by the density of the internal standard.

Effects of Fire Injury on Mountain Pine Beetle Entry Ten separate trees from each of the four fire injury categories were selected for a beetle entry bioassay that was conducted under no-choice conditions. Beetles were captured using ten 12-funnel flight traps (Lindgren, 1983) that were baited with *trans*-verbenol, myrcene, and *exo*-brevicomin from Contech Enterprises Inc. Captured beetles were kept in a refrigerator for no longer than 1 wk prior to bioassay. Only vigorous, active female beetles were used. A 13 cm^2 square of aluminum screening was secured to the north or unburned portion of the tree bole at 1.3 m high. Five females were introduced into each cage. Needles and lichen from lodgepole pine branches were provided to give the beetles traction on a natural surface. Beetle entry and mortality were tabulated after 24 hr. For each tree, we noted diameter at 1.3 m, height, and potential sources of stress to incorporate conditions other than fire injury category that may affect beetle entry in our analysis, as described previously. The trees included in this experiment were of similar size and condition as those analyzed for chemical content.

Statistical Analyses Concentrations of volatiles were calculated on a per dry weight of phloem basis. Concentrations from the north and south, or burned and unburned, aspects of the bole were averaged. We did not observe any

difference between burned or unburned bole portions for either treatment, and we only observed differences between the north and south aspects of the bole for constitutive, unburned samples, and induced low injury samples (Supplementary Materials: Table 1). Concentrations were square root transformed to achieve normality. Normality and homogeneous variance were tested using quantile-quantile plots and Bartlett tests, respectively. We performed a two-way ANOVA using the statistical program R v2.7.2 (R Core Development Team, 2008) to identify potential differences of total volatile amounts among fire injury categories, constitutive or induced treatment, and the interaction between category and treatment. We used ANOVA to test for differences across fire injury categories within constitutive and induced phloem, and then performed *t*-tests with a Bonferroni correction to test for pairwise differences. Separate two-way ANOVAs tested for potential differences of total concentrations between fire injury category and bole aspect, and between constitutive or induced treatment and bole aspect. We constructed a single generalized linear model with a square-root transformation to evaluate other potentially important pathogenic and environmental variables on total concentrations. We used a backward elimination procedure with Akaike's information criteria (AIC) (Akaike, 1973), and selection of variables with $\alpha \leq 0.05$, to identify the best model for analyzing total volatile concentrations.

Individual volatile percentages in each sample were calculated and compared among fire-injury categories and constitutive and induced treatments. Normality was not achieved after an arcsin square-root transformation, so tests were performed using the non-parametric Kruskal-Wallis rank-sum test.

Beetle entry into trees of various fire-injury categories was analyzed using a *Chi-square* analysis. We constructed a single generalized linear model with a logit transformation to evaluate variables other than fire injury on no-choice, beetle entry. We used a backward eliminated procedure with AIC (Akaike, 1973), and selection of variables with $\alpha \leq 0.05$, to identify the best model for no-choice, beetle entry analysis.

Results

Effects of Fire Injury on Constitutive and Induced Volatiles We detected 13 monoterpene hydrocarbons in the tissue of the constitutive samples, 12 monoterpene hydrocarbons in the induced samples, 4 allylic monoterpene alcohols, 1 ester and 1 phenyl propanoid, of which the monoterpene hydrocarbons comprised at least 96% in all induction and fire injury categories (Tables 1 and 2). Hereafter, our use of 'total volatiles' refers to these compounds. Total volatile concentrations (mg/g dry phloem)

Table 1 Effects of fire injury on percentages of individual monoterpenes and additional volatiles in constitutive phloem tissue of lodgepole pine, by Kruskal-Wallis rank sum

Compound	Unburned	Low	Moderate	High	χ^2	*df*	*P*
(−) α-Pinene	2.55±0.157	3.60±0.140	3.58±0.231	5.25±0.370	60.8	3	<0.001
(+) α-Pinene	0.720±<0.100	1.30±0.726	1.05±0.109	1.57±0.155	30.5	3	<0.001
Myrcene	3.96±0.470	3.76±0.335	2.94±0.351	1.73±0.229	41.1	3	<0.001
(−) Camphene	<0.100±<0.100	<0.100±<0.100	<0.100±<0.100	<0.100±<0.100	2.69	3	0.442
(+) Camphene	<0.100±<0.100	<0.100±<0.100	<0.100±<0.100	<0.100±<0.100	9.94	3	0.019
(+) 3-Carene	0	<0.100±<0.100	0	0	3.04	3	0.385
(+) β-Pinene	12.0±1.07	7.48±0.749	7.68±0.982	10.9±1.14	22.1	3	<0.001
α-Terpinene	<0.100±<0.100	<0.100±<0.100	0	<0.100±<0.100	2.00	3	0.573
(−) β-Pinene	10.9±0.796	14.2±0.757	13.8±1.03	17.4±1.27	16.4	3	<0.001
(+) Limonene	<0.100±<0.100	0.156±<0.100	0.275±<0.100	0.782±0.118	55.2	3	<0.001
β-Phellandrene	64.5±1.29	64.3±1.25	64.4±1.55	56.7±1.72	14.6	3	0.002
Terpinolene	1.90±0.224	1.61±0.215	1.50±0.310	0.403±<0.100	38.4	3	<0.001
Linalool	<0.100±<0.100	<0.100±<0.100	<0.100±<0.100	<0.100±<0.100	5.15	3	0.161
4-Allylanisole	1.05±0.178	0.551±<0.100	0.371±<0.100	0.236±<0.100	28.2	3	<0.001
α-Terpineol	0	<0.100±<0.100	<0.100±<0.100	0.365±0.198	8.45	3	0.038
Borneol	<0.100±<0.100	<0.100±<0.100	0.127±<0.100	<0.100±<0.100	3.92	3	0.270
Geraniol	<0.100±<0.100	<0.100±<0.100	0.201±0.115	<0.100±<0.100	3.88	3	0.275
(−) *trans*-Caryophyllene	2.16±0.404	1.77±0.261	2.77±1.09	<0.100±<0.100	61.4	3	<0.001
Bornyl Acetate	<0.100±<0.100	0	0	0	2.96	3	0.398

Table 2 Effects of fire injury on percentages of individual monoterpenes and additional volatiles in induced phloem tissue of lodgepole pine, by Kruskal-Wallis rank sum

Compound	Unburned	Low	Moderate	High	χ^2	*df*	*P*
(−) α-Pinene	3.21±0.800	3.78±0.140	4.29±0.280	5.57±0.432	46.9	3	<0.001
(+) α-Pinene	1.69±<0.100	1.69±<0.100	1.54±0.139	1.66±0.177	1.71	3	0.634
Myrcene	2.58±0.131	2.95±0.194	2.48±0.321	1.66±0.264	52.1	3	<0.001
(−) Camphene	<0.100±<0.100	0.153±<0.100	<0.100±<0.100	0	22.7	3	<0.001
(+) Camphene	<0.100±<0.100	0.145±<0.100	<0.100±<0.100	0	24.1	3	<0.001
(+) β-Pinene	14.7±1.09	13.5±1.00	9.92±1.09	11.6±1.28	20.3	3	<0.001
(−) β-Pinene	9.23±0.638	11.8±0.940	14.0±1.07	18.3±1.39	24.9	3	<0.001
(+) Limonene	<0.100±<0.100	0.288±<0.100	0.598±0.126	0.600±0.144	24.3	3	<0.001
β-Phellandrene	65.5±1.03	63.0±0.956	63.2±1.62	54.3±2.19	19.4	3	<0.001
Terpinolene	1.32±0.107	1.09±0.112	0.662±0.136	0.133±<0.100	111	3	<0.001
Linalool	0	<0.100±<0.100	<0.100±<0.100	0	43.0	4	<0.001
4-Allylanisole	0.676±<0.100	0.538±<0.100	0.212±<0.100	0.218±<0.100	81.7	3	<0.001
α-Terpineol	<0.100±<0.100	0.116±<0.100	0.158±0.118	0	5.68	3	0.128
Borneol	0	<0.100±<0.100	<0.100±<0.100	0.132±0.104	13.5	3	0.004
Geraniol	<0.100±<0.100	<0.100±<0.100	<0.100±<0.100	0	6.70	3	0.082
(−) *trans*-Caryophyllene	0.679±0.226	0.697±0.129	0.605±0.184	<0.100±<0.100	40.8	3	< 0.001
Bornyl Acetate	<0.100±<0.100	<0.100±<0.100	<0.100±<0.100	<0.100±<0.100	0.878	3	0.831
trans-β-Ocimene	0	0	0	<0.100±<0.100	3.21	3	0.361

were affected by fire injury category, induction treatment, and the interaction between category and treatment (category: $F_{(3,\ 391)}$=8.65, P<0.001, treatment: $F_{(1,391)}$=67.9, P<0.001, interaction: $F_{(3,391)}$=4.73, P=0.003). Total concentrations of volatiles within constitutive phloem tissue did not vary with fire injury ($F_{(3,196)}$=1.04, P=0.376). In contrast, the concentrations of induced volatiles decreased by nearly half with the occurrence of any fire injury, regardless of severity (Fig. 1, $F_{(3,195)}$=18.1, P<0.001). This pattern did not vary among the various measurements of fire injury.

The linear regression model incorporating all tree- and stand-level factors that yielded the best fit was R^2=0.263, $F_{(11,387)}$=12.6, P<0.001, and included fire injury category, induction treatment, the interaction between category and treatment, tree height, hillside slope steepness, and UTM-easting and UTM-northing plot position (Table 3). In addition to the effects of fire injury and induction, total volatile concentrations decreased with tree height, slope steepness, and more eastern and northern plot positions (Supplementary Material: Table 2).

Effects of Fire Injury on Constitutive and Induced Volatile Composition Fire injury influenced the proportions of several volatiles in constitutive phloem tissue (Table 1). Concentrations (mg/g dry phloem) are listed in Supplementary Material: Tables 3 and 4. Proportions of (−) α-pinene, (+) α-pinene, (−) β-pinene, (+) limonene, and α-terpineol increased with fire injury by 3%, 1%, 7%, 0.6%, and 0.3%, respectively. Proportions of myrcene, β-phellandrene, terpinolene, 4-allylanisole, and (−) *trans*-caryophyllene decreased with fire injury, by 2%, 7%, 1%, 0.8%, and 2%, respectively. Two monoterpenes showed a non-linear response to fire injury: (+) 3-Carene was detected only in the low fire injury category, and (+) β-pinene was

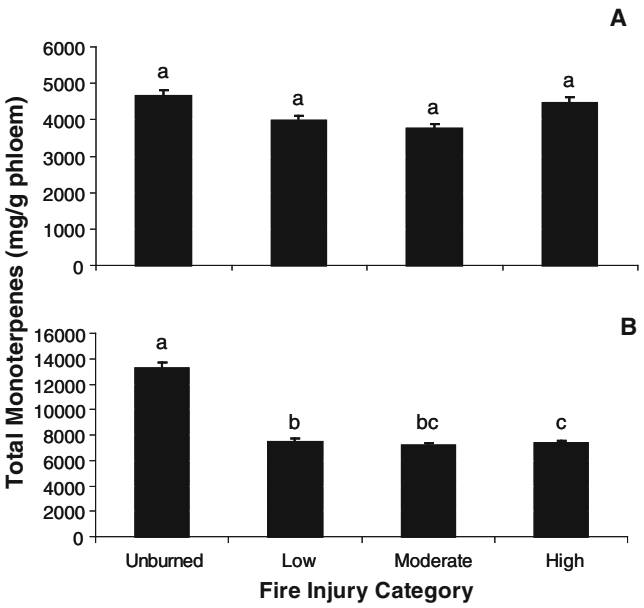


Fig. 1 Effect of wildfire injury on concentrations of total monoterpenes and additional volatiles in lodgepole pine phloem. **a**. Constitutive tissue ($F_{(3,196)}$=1.04, P=0.376). **b**. Induced tissue ($F_{(3,195)}$=9.59, P<0.001). Means with different letters are statistically different at P<0.05

Table 3 Effects of fire injury category, induction by fungal symbionts of mountain pine beetle, and additional tree- and site-level variables on total content of monoterpenes and additional volatiles in lodgepole pine phloem

Variable	Estimate (**x**)	Std. Error	*P*
β_o (intercept; unburned category and constitutive treatment)	2.27×10^5	6.98×10^4	0.001
β_1 (low category)	−17.6	6.69	0.009
β_2 (moderate category)	−34.4	9.63	<0.001
β_4 (high category)	−20.0	8.21	0.015
β_5 (induced treatment)	35.6	5.87	<0.001
β_6 (tree height)	−0.783	0.302	0.010
β_7 (hillside slope steepness)	−0.827	0.294	0.005
β_8 (UTM – easting)	−0.022	0.008	0.009
β_9 (UTM – northing)	−0.044	0.013	0.001
β_{10} (low category, induced treatment interaction)	−29.6	8.30	<0.001
β_{11} (moderate category, induced treatment interaction)	−16.5	8.32	0.048
β_{12} (high category, induced treatment interaction)	−24.3	8.30	0.004

Plot locations (UTM zone 12). 1: 548328 easting, 4938992 northing; 2: 548380 easting, 4938563 northing; 3: 548432 easting, 4938674 northing; 4: 548502 easting, 4938749 northing; 5: 548215 easting, 4938187 northing; 6: 548311 easting, 4938616 northing; 7: 549591 easting, 4937979 northing; 8: 548312 easting, 4938455 northing; 9: 548367 easting, 4938992 northing

Multiple Linear Regression Model Was Selected Based on Best Fit: $\mathbf{y} = \beta_0 + \beta_1\mathbf{x_1} + \ldots + \beta_k\mathbf{x_k}$. $R^2 = 0.263$, $F_{(11,387)} = 12.6$, $P < 0.001$

highest in the unburned category and lowest in the low fire injury category. (−) Camphene, (+) camphene, α-terpinene, linalool, borneol, geraniol, and bornyl acetate showed no change in percentage with fire injury.

Fire injury also affected the percentages of individual compounds within induced phloem tissue (Table 2). Proportions of the monoterpenes (−) α-pinene, (−) β-pinene, and (+) limonene increased with fire injury, by 2%, 9%, and 0.6%, respectively. Proportions of myrcene, β-phellandrene, 4-allylanisole, and (−) *trans*-caryophyllene decreased with fire injury by 1%, 11%, 0.4%, and 0.6%, respectively. (+) Camphene was highest in the low injury category but not detected in the high injury category. (−) Camphene likewise was not detected in the high injury category. (+) β-Pinene was highest in the unburned category and lowest in the moderate category. Linalool was detected only in the low and moderate injury categories. Terpinolene was highest in the high fire injury category and lowest in the moderate category. Borneol was not detected in uninjured trees, and geraniol was not detected in the highly-injured trees. Percentages of (+) α-pinene, α-terpineol, bornyl acetate, and *trans*-β-ocimene in induced tissue were not affected by fire injury.

Effects of Fire Injury on Mountain Pine Beetle Entry There was no statistical difference among fire injury categories in the percentage of trees entered by caged female mountain pine beetles (Fig. 2a, χ^2=3.51, *df*=3, *P*= 0.320), nor in the percentage of beetles that entered these trees (Fig. 2b, χ^2= 4.58, *df*=3, *P*>0.250). The overall trend, however, was of a parabolic relationship, with highest entry into lightly injured trees. Incorporating other variables did not yield statistically significant explanatory variables of mountain pine beetle entry (Table 4, R^2=0.012, $F_{(3,36)}$=1.15, *P*=0.341).

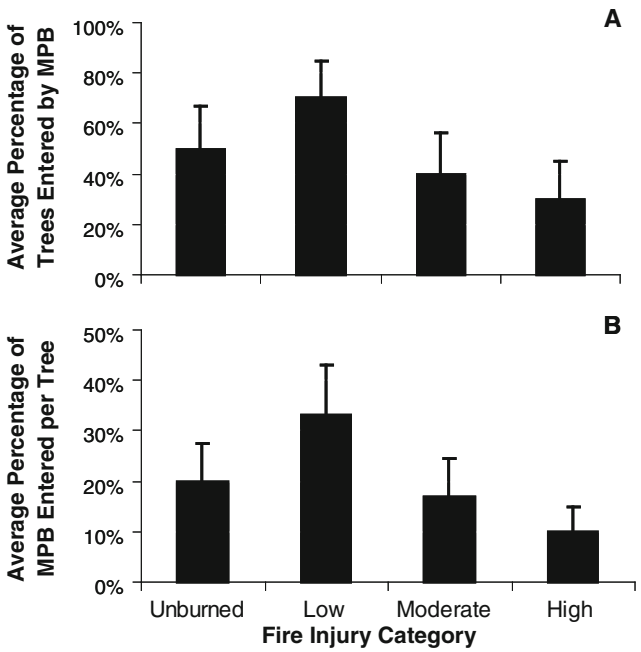


Fig. 2 Entry of caged female mountain pine beetles (MPB) into lodgepole pines that had experienced varying degrees of wildfire injury. **a**. Average percentage of lodgepole pines entered by mountain pine beetles (χ^2=3.51, *df*=3, *P*=0.320). **b**. Average percentage of mountain pine beetles entered per tree (χ^2=4.58, *df*=3, *P*>0.250)

Table 4 Linear regression model, with logit transformation, relating incidence of mountain pine beetle entry to fire injury category

Variable	Estimate (**x**)	Std. Error	*P*
β_o (intercept; unburned category)	0.500	0.159	0.003
β_1 (low category)	0.200	0.225	0.380
β_2 (moderate category)	−0.100	0.225	0.659
β_3 (high category)	−0.200	0.225	0.380

Model Was Selected Based on Best Fit by R^2. These Results Are Fitted to Equation, $\ln(\mu/1-\mu) = \beta_0 + \beta_1 \mathbf{x_1} + \ldots + \beta_k \mathbf{x_k}$. $R^2 = 0.012$, $F_{(3,36)} = 1.15$, $P = 0.341$

Discussion

These results indicate that fire injury reduced the induced, but not constitutive quantities of total monoterpenes and other less abundant volatiles available to defend against attack by mountain pine beetles and its associated micro-organisms. Further, only a small amount of injury was needed to exert this effect. This reduction in the ability to undergo induced accumulation of monoterpenes and additional volatiles at an attack site corresponds with increased likelihood of successful attack by mountain pine beetle in lodgepole pines injured by wildfire in this same region (Powell et al., 2012). Induced accumulation of monoterpenes likewise has been shown to be compromised by a broad range of biotic and abiotic stress agents in multiple conifer-bark beetle systems (Reviewed in Raffa et al., 2005). Similarly, a recent study by Boone et al. (2011) identified induced monoterpene accumulation as the best overall predictor of lodgepole pine defense against mountain pine beetle.

Fire injury also affected the proportions of individual compounds within trees. Among the volatiles affected in constitutive, induced, or both phloem tissues, (−) α-pinene, (+) limonene, myrcene, 4-allylanisole, and terpinolene, are known to affect several aspects of bark beetle colonization, such as entry (Coyne and Lott, 1976), survival (Raffa and Berryman, 1983), and pheromone communication (Wood, 1982; Hayes and Strom, 1994; Erbilgin et al., 2007; Borden et al., 2008; Blomquist et al., 2010). 4-Allylanisole inhibits mountain pine beetle aggregation (Hayes and Strom, 1994), so its reduction in both constitutive and induced phloem tissue with fire injury potentially could create an environment more conducive for mountain pine beetle success. Likewise, the increase in (−) α-pinene with fire injury could benefit the beetle, as this compound is a precursor of mountain pine beetle aggregation pheromone during biosynthesis (Wood, 1982). However, the relative reductions in myrcene and terpinolene with fire injury could have the opposite effect, as both of these compounds synergize the attractiveness of the mountain pine beetle's aggregation pheromone (Borden et al., 2008). Overall, the compositional changes in individual volatiles caused by fire injury appeared potentially more important in constitutive, rather than induced phloem tissue. We currently lack sufficient information with which to characterize these proportionate changes as programmed tree reactions to specific agents that predispose them to bark beetle attack, generalized stress reactions, beetle adaptations to chemical changes that accompany stress, idiosyncratic events, or various combinations thereof. For example, Boone et al. (2011) saw some of these same changes, such as increased α-pinene and reduced β-phellandrene, in lodgepole pine following infestation by lower stem beetles, but other reactions differed.

Our caged beetle entry results showed no significant difference among fire injury categories, but identified a slight trend of more entry in the low injury category. This pattern is interesting because several studies have shown the incidence of bark beetle attack is often higher in moderately burned than in either unburned or severely burned trees (Elkin and Reid, 2004; Powell et al., 2012). We cannot discount the possibility that larger sample sizes would have generated a statistically significant relationship, and so additional research is necessary. Additionally, future research should evaluate the relative ability of entered beetles in healthy vs. fire-injured trees to elicit attraction in the field.

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borer than evolutionarily naïve North American ash species (Rebek et al., 2008).

Relatively little is known about traits that underlie resistance of woody angiosperms to wood-boring insects. Proposed physical mechanisms of resistance include high rates of sap flow (Morewood et al., 2004) and specialized water-containing vesicles (Hanks et al., 1991, 1999), which can inhibit larval establishment. Additionally, induction of wound periderms (callus tissues) in response to larval feeding is hypothesized to function defensively by encapsulating larvae (Anderson, 1944; Dunn et al., 1990; Fierke and Stephen, 2008). Putative chemical mechanisms of resistance include constitutive and induced secondary metabolites present in bark and phloem tissues (Morewood et al., 2004). For example, differences in phenolic profiles in stem phloem tissues of resistant and susceptible ash species were implicated as resistance mechanisms to emerald ash borer (Eyles et al., 2007).

The secondary chemistry of *Betula* is dominated by phenolic compounds, including low molecular weight phenolics, tannins, and lignin (Julkunen-Tiitto et al., 1996; Ossipov et al., 1996; Mämmelä, 2001; Laitinen et al., 2004; Mattson et al., 2005), but how these chemicals vary in the phloem of mature paper birch and European white birch is not known. Therefore, as a follow-up to the common-garden study of Nielsen et al. (2011) that documented greater resistance to BBB in paper birch relative to European white birch, we initiated a study at the same site with the goal of elucidating potential mechanisms underlying variation in resistance. Specifically, we compared qualitative and quantitative differences in phenolic profiles of the stem phloem tissues of these two species. The first objective was to compare interspecific variation of constitutively expressed low molecular weight phenolics present in phloem tissues of paper birch and European white birch and to assess how this variation corresponds with reported patterns of resistance to BBB. The second objective was to analyze intraspecific variation in constitutively expressed phenolics among clones and/or half-siblings within each species.

Methods and Materials

Plant Material and Experimental Design Paper birch and European white birch were grown in a common garden for 12 years at the Ohio Agricultural Research and Development Center in Wooster, OH, USA. Trees were planted at a 3×3 m spacing. In total, there were 56 paper birch and 54 European white birch. Paper birch and European white birch had mean stem heights of 35.3±1.3 (SE) and 46.5±1.2 m, and mean stem diameters of 9.1±0.3 and 10.4±0.3 cm, respectively. All trees were reproductively mature and had similar phenology, with bud burst of all individuals varying by less than 2 weeks (V.L. Muilenburg, personal observations).

Intraspecific variation of paper birch was assessed by using two clones (231 and M2) and two half-sibling families (M1 and 12). M1, M2, and 12 were collected from Midland County Michigan, and 231 were collected from Pennsylvania. Intraspecific variation of European white birch was assessed by using four clones (5818, 5832, 5938 and 50076) selected by the Finnish Forest Research Institute. These European white birch clones are used in birch cultivation by the Finnish forest industry and have been evaluated previously for their resistance to mammalian and insect herbivores (Rousi et al., 1997; Ylioja et al., 2002).

Trees were planted in a randomized complete block design with 8 trees of each species per block, two replicates per clone or half-sibling in each block. One tree of each of the clones or half-siblings per species was randomly sampled in each of the 8 blocks (N=64 trees). Clone/half-sibling was treated as a factor nested within species.

Phloem Samples and Extraction One phloem sample 10 mm diam was excised from each tree stem approximately 1.3 m above ground level with an arch punch. Samples were taken 26 June 2007 to correspond with the natural timing of feeding by BBB larvae (Akers and Nielsen, 1984). The arch punch was inserted into the tree to the depth of the phloem-xylem interface in order to excise the phloem and outer bark. Upon excision, the outer bark was removed, and the phloem samples were flash frozen immediately in liquid nitrogen. The phloem was ground to a powder in liquid nitrogen, and 100 mg (fresh weight) were extracted twice in 500 µl of HPLC-grade methanol (Fisher, Pittsburgh, PA, USA) for 24 h at 4°C (Bonello and Blodgett, 2003). The supernatants were combined and stored in 1.5 ml microcentrifuge tubes at −20°C until analysis by high-performance liquid chromatography (HPLC).

Qualitative Phenolic Analysis Constitutively expressed phenolics were characterized with HPLC-MS analysis by using a Varian 212-LC chromatography pump, Varian Polaris™ RP18, 5 µm, 4.6×150 mm column, ProStar 410 HPLC Autosampler, and Varian 500-MS ion trap mass spectrometer with the ESI source operated in the negative ion mode. The MS detector had a capillary voltage of 80 V and a cone voltage of 5000 V, with a scan range of m/z 100–1400. MS/MS scans were completed on-the-fly by the instrument when the threshold of the pseudomolecular ion reached 20,000 counts. Ions generated by MS^2 and MS^3 underwent further fragmentation at 4,000 counts. The binary mobile phase consisted of 0.1% glacial acetic acid

in HPLC-grade water (solvent A) and 0.1% glacial acetic acid in HPLC-grade methanol (solvent B). The flow rate was 300 μl/min with a linear gradient of B from 0 to 10% in 10 min, then to 25% by 20 min, to 35% by 40 min, to 45% by 56 min, to 65% by 64 min, and finally to 100% by 70 min, with a 20 min hold. The injection volume for each sample was 5 μl.

Quantitative Phenolic Analysis Phenolics were quantified with a Waters 2690 separations module (Milford, MA, USA) equipped with a Waters Xterra™ RP18, 5 μm, 4.6× 150 mm column, a Waters Xterra™ RP18, 3.9 μm, 3.0× 20 mm guard column, and a Waters 996 photodiode array detector. The binary mobile phase consisted of 2% glacial acetic acid in HPLC-grade water (solvent A) and 2% glacial acetic acid in HPLC-grade methanol (solvent B). The flow rate was 1 ml/min, with a linear gradient of B: 0% (0–2 min), 0–10% (2–4 min), 10–48% (4–20 min), 48–100% (20–38 min), and then held for 1 min. The injection volume for each sample was 10 μl.

Phenolics were quantified at 280 nm against the following standards: catechin (Alexis Biochemicals, San Diego, CA, USA), chlorogenic acid (MP Biomedicals, Solon, OH, USA), and salicin (Sigma Aldrich, Milwaukee, WI, USA). The glycosides catechin pentoside, betuloside, betuloside pentosides, and diarylheptanoids were expressed as salicin equivalents. Catechin and procyanidins were expressed as catechin equivalents. Coumaroylquinic acid, platyphylloside, and unknown peaks 9–12 were expressed as chlorogenic acid equivalents.

To align the UV and MS chromatograms, which required different solvent programs, a subset of samples was re-analyzed on an in-line HPLC/UV/MS instrument: a Waters 2695 HPLC (Milford, MA, USA) with a 2.6 μm× 3.00 mm×150 mm Kinetex™ RP18 column and Krud-Katcher™ Ultra filter (Phenomenex, Torrance, CA, USA), a Waters 996 photodiode array detector, and a Micromass ZQ 2000 quadrupole mass detector operated in the negative ion ESI mode. UV absorbance was determined from 237 nm to 400 nm. The MS detector had a capillary voltage of 3.2 kV, a cone voltage of 60 V, and a source temperature of 80°C, with a scan range of m/z 100–1200. The autosampler and column temperatures were set at 30°C. Samples were run using the solvent parameters for the UV quantitation above, with the flow split 2:1 to reduce the rate of mobile phase entering the ZQ detector. When appropriate, the additional fragmentation from the quadrupole spectra were used to help with compound identification.

*Statistical Analyses*The relative concentrations of phenolic compounds were analyzed by univariate analysis of variance (ANOVA) using SAS 9.1 (SAS Institute 1999). All factors (block, species, clone/half-sibling) were considered fixed. Data that did not have equal variances were log-transformed. Fisher's LSD was used to separate means of clones and half-siblings (α=0.05). Intraspecific and interspecific variation of phenolic profiles was analyzed by using principal component analysis (PCA) with Minitab 15 (Minitab Inc. 2007).

Results

Characterization of Phenolics Seventeen compounds, which appeared in at least half of the samples for either species, were quantified (Fig. 1). Characterization of these chemicals was based on comparison of UV spectra, mass fragmentation, and relative retention time with published data and standards, when available (Table 1).

Monomeric/Oligomeric Flavanols Peak 1 fragmentation patterns $[M\text{-}H\text{-}152\text{-}18]^-$ are indicative of retro-Diels-Alder fission (RDA-F) of heterocylic rings with the loss of water (Friedrich et al., 2000; Shui and Leong, 2004). These spectral characteristics are consistent with those reported for procyanidin dimers (Tsang et al., 2005). Similarly, peak 4 fragmentation patterns $[M\text{-}H\text{-}170]^-$ are indicative of RDA-F with the loss of water as well as interflavan bond cleavage $[M\text{-}H\text{-}288]^-$ (Friedrich et al., 2000; Shui and Leong, 2004). These spectral characteristics are consistent with those reported for a procyanidin trimer (Tsang et al., 2005). Regio- and stereochemistry of the interflavan bonds were not determined for peaks 1 and 4. Peak 2 fragment ions (*m/z* 421.2, 289.1, and 245) correspond with those reported for catechin pentoside previously found in European white birch wood (Mämmelä, 2001), and after the loss of pentose, with fragmentation patterns of a catechin standard. Peak 3 was identified as catechin by comparison with a standard.

Phenolic Acid Peak 5 had a pseudomolecular ion of *m/z* 337.1 and a λ_{max} of 311 nm, which are consistent with coumaroylquinic acid (Ossipov et al., 1996; Poon, 1998).

Phenolic Arylbutanoids Peaks 6 and 8 ($[M\text{-}H]^-$=459.3) had the same fragmentation patterns in the quadrupole mass spectrometer, suggesting that they are enantiomers. Fragmentation patterns $[M\text{-}H\text{-}132]^-$ indicate a loss of pentose, and the remaining aglycone (*m/z* 327.2) is consistent with betuloside (Mämmelä, 2001). Therefore, compounds 6 and 8 were tentatively identified as betuloside pentoside A and betuloside pentoside B, respectively. Peak 7, with $[M\text{-}H]^-$= 327.2, is consistent with betuloside (Mämmelä, 2001).

Phenolic Diarylheptanoids MS^n of peak 13 ($[M\text{-}H]^-$=475) did not generate any daughter ions; however, the quadrupole mass spectrometer generated a base ion at *m/z* 295,

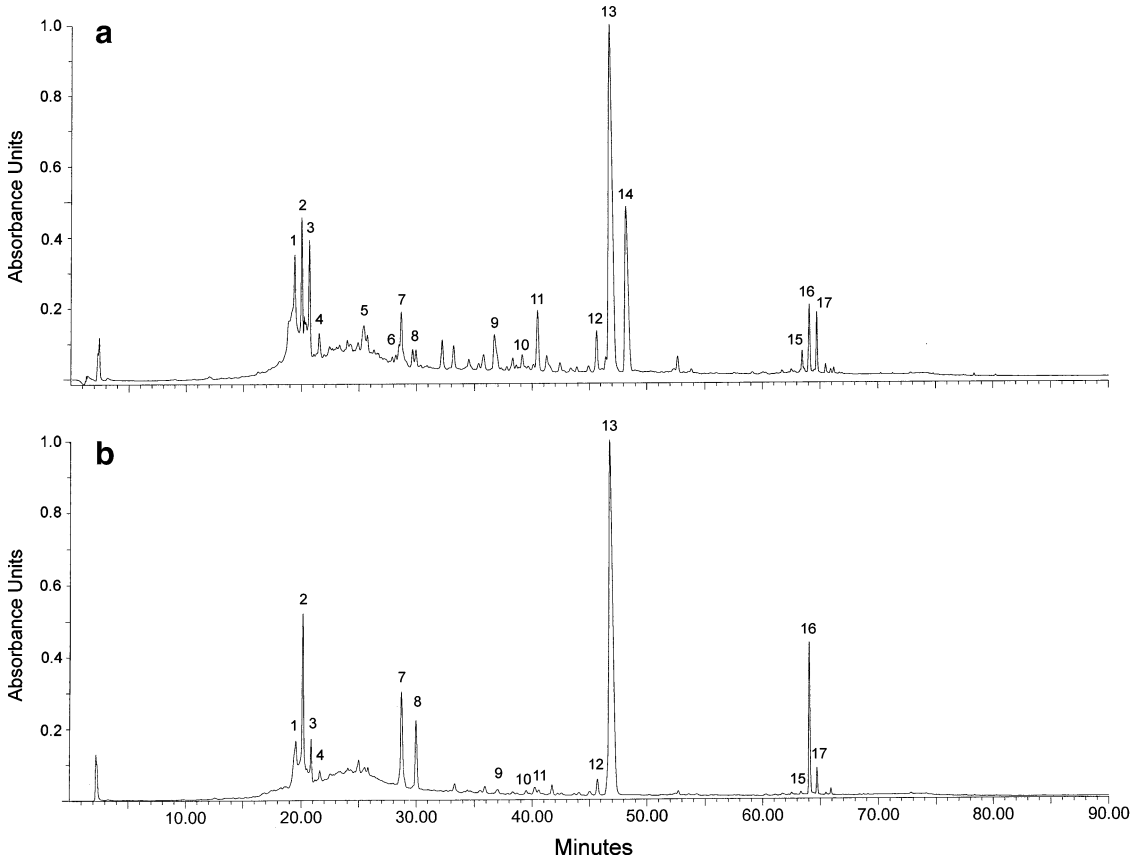


Fig. 1 Comparison of constitutive phenolic profiles from stem phloem tissues of (**a**) paper birch, *Betula papyrifera* and (**b**) European white birch, *Betula pendula*. Chromatograms were generated from the HPLC-PDA at 280 nm. For characterization of numbered peaks, see Table 1

indicating loss of hexose and water, consistent with platyphylloside (Vainiotalo et al., 1991; Smite et al., 1993) (Fig. 2a). Major fragments of peak 14 (*m/z* 149.1 and 121.0) and the aglycone (*m/z* 315.3) are consistent with a diarylheptanoid hexoside (Fig. 2b) related to platyphylloside (Jiang et al., 2006). The MS of peaks 15, 16, and 17 suggest the same aglycone, but with different sugar moieties (Fig. 2c). *M/z* 299.3 and its daughter *m/z* 121 are consistent with an ethyl *p*-hydroxybenzoyl moiety (Jiang et al., 2006) and the diarylheptanoid centrolobol (Smite et al., 1993). Peak 15 fragmentation $[M\text{-}H\text{-}132\text{-}132\text{-}162]^-$ suggests the presence of a hexose and two pentose sugars, while peak 16 fragmentation $[M\text{-}H\text{-}132\text{-}162]^-$ is consistent with a hexose and a pentose sugar, and peak 17 fragmentation $[M\text{-}H\text{-}162]^-$ indicates a hexoside. The aglycone of centrolobol has been detected previously in European white birch phloem, conjugated with a monosaccharide, disaccharide, and trisaccharide comprised of glucose and apiose (Smite et al., 1993).

Unknowns Four of the 17 selected compounds (peaks 9–12) were not given tentative identifications due to lack of correspondence of diagnostic mass and UV spectra to that of known chemical species.

*Interspecific Variation*There was both qualitative and quantitative variation in phenolics between species (Table 2). Paper birch phloem contained several compounds not detected in European white birch phloem including coumaroylquinic acid (5), betuloside pentoside A (6), and diarylheptanoid hexoside (14). Paper birch phloem also had higher concentrations of six phenolics than European white birch phloem, including betuloside pentoside B (8) ($F_{1,\ 48}$= 20.86), centrolobol glycoside (15) ($F_{1,\ 48}$=20.72), and four unknown compounds [(9, 10, 11, 12) ($F_{1,\ 48}$= 38.87, 68.97, 32.44, and 8.74, respectively)]. European white birch phloem contained higher concentrations of three phenolics than paper birch phloem, including catechin pentoside (2) ($F_{1,\ 48}$= 62.97), platyphylloside (13) ($F_{1,\ 48}$=12.26), and centrolobol glycoside (16) ($F_{1,\ 48}$=31.99).

The phenolic profiles of paper birch and European white birch were different (Fig. 3), as indicated by principal component analysis. Separation between species occurred largely along the first PC axis, which explained 29.6% of

Table 1 Characterization of constitutive phenolic chemistry of stem phloem tissue of paper birch, *Betula papyrifera*, and European white birch, *Betula pendula*, using ion trap mass spectrometry

Peak No.	Rt (min)	$[M-H]^-$ (m/z)	MS^2 Ions	MS^3 Ions	MS^4 Ions	λ_{max} (nm)	Tentative Identification	Presence in Tree Species	References Used for Identification
1	19.4	577.3	**425.2**, 451.1, 289.3	407.1	281.1, 283.1, 255.1	280	procyanidin dimer	both	Tsang et al. 2005
2	20.0	421.2	289.1	**245.1**[a], 205.1	203.1	279	catechin pentoside	both	Mämmelä 2001
3	20.7	289.1	245.1	**203.0**, 187.1	175.0, 187.9, 161.0	280	catechin	both	Mämmelä 2001
4	21.6	865.4	**695.4**, 577.4, 738.9	543.2, 451.3		278, 283sh	procyanidin trimer	both	Tsang et al. 2005
5	25.4	337.1	163	118.9	92.9	284sh,311	coumaroylquinic acid	*B. papyrifera*	Poon 1998
6	28.2	459.3				278	betuloside pentoside A	*B. papyrifera*	Mämmelä 2001
7	28.7	327.2				278	betuloside	both	Smite et al. 1993; Mämmelä 2001
8	29.9	459.3	327.2	165.1	121.0, 106.0	278	betuloside pentoside B	both	Mämmelä 2001
9	36.7	487.2				280	unknown	both	
10	39.2	427.2	293.1	**125.0**, 233.1, 191.0	97	280	unknown	both	
11	40.5	311.2	205.1	**120.9**, 146.9	92.9	280	unknown	both	
12	45.6	607.4	310.6, 477.3			280	unknown	both	
13	46.7	475				278, 283sh	platyphylloside	both	Smite et al. 1993; Mämmelä 2001
14	48.2	477.3	315.3	**149.1**, 147.1, 297.1	107.0, 121.0, 92.9	278, 283sh	diarylheptanoid hexoside	*B. papyrifera*	Jiang et al. 2006
15	63.5	725.5	**593.5**, 461.6	461.4	299.3	278, 283sh	centrolobol + hexose + pentose + pentose	both	Smite et al. 1993
16	64.1	593.4	**461.4**, 299.5	299.3	177.2, 121.1	278, 283sh	centrolobol + hexose + pentose	both	Smite et al. 1993
17	64.7	461.4	299.3	**177.1**, 121.1	121.0, 106.0, 159.1	278, 283sh	centrolobol hexoside	both	Smite et al. 1993

[a] Bold indicates ions that underwent further fragmentation

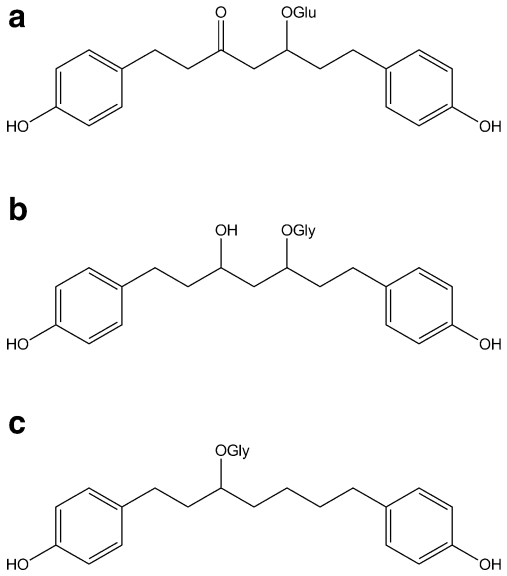


Fig. 2 Proposed identity of HPLC peaks (Fig. 1). (**a**) platyphylloside (13), (**b**) diarylheptanoid hexoside (14), and (**c**) centrolobol glycosides containing three, two and one sugar moieties (15, 16, and 17, respectively)

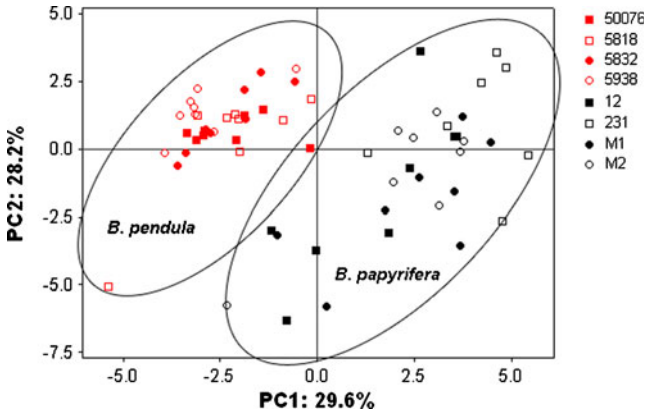


Fig. 3 Inter- and intraspecific variation in phenolic chemistry from stem phloem tissues of European white birch, *Betula pendula*, and paper birch, *Betula papyrifera*, as indicated by principal component analysis (PCA). 50076, 5818, 5832, and 5938 are clones of European white birch. 231 and M2 are clones and 12 and M1 are half-siblings of paper birch. Percentage of total variability explained by first two principal components shown

the total variation. Location along the first PC axis was primarily determined by coumaroylquinic acid (5) (eigenvector (EV): 0.388), diarylheptanoid hexoside (14) (EV: 0.391), and compound 10 (EV: 0.376), which were all associated with paper birch, and catechin pentoside (2) (EV: −0.291) and centrolobol glycoside (16) (EV: −0.214), which were associated with European white birch.

Intraspecific Variation Within each species, there were quantitative differences among clones and/or half-siblings (Table 3). Among clones and half-siblings of paper birch, there was variation in concentrations of four out of 17 compounds: catechin pentoside (2) ($F_{6,\ 48}$=5.26), procyanidin trimer (4) ($F_{6,\ 48}$=16.60), coumaroylquinic acid (5) ($F_{6,\ 48}$=4.49), and betuloside (7) ($F_{6,\ 48}$=9.14). Among clones of European white birch there was variation in concentrations of five out of 14 compounds: catechin (3) ($F_{6,\ 48}$=3.42), procyanidin trimer (4) ($F_{6,\ 48}$=4.33), compound 12 ($F_{6,\ 48}$=3.29), and two centrolobol glycosides (15, 16) ($F_{6,\ 48}$=13.81, 5.18, respectively).

Table 2 Interspecific comparison of constitutive phenolic[a] concentrations in stem phloem tissue of paper birch, *Betula papyrifera*, and European white birch, *Betula pendula*

Peak No.	Compound	*B. papyrifera*	*B. pendula*	*P*-value
1	procyanidin dimer	6.78±0.30	6.52±0.19	0.380
2	catechin pentoside	62.07±4.97	108.9±4.1	<0.0001
3	catechin	12.53±0.75	13.44±0.52	0.354
4	procyanidin trimer	10.88±0.80	11.18±0.85	0.941
5	coumaroylquinic acid	3.36±0.30	ND[b]	
6	betuloside pentoside A	12.23±1.19	ND	
7	betuloside	28.74±3.42	28.13±1.25	0.617
8	betuloside pentoside B	35.18±3.17	21.39±1.54	<0.0001
9	unknown	4.71±0.57	1.75±0.18	<0.0001
10	unknown	2.62±0.31	0.16±0.12	<0.0001
11	unknown	2.22±0.22	0.67±0.19	<0.0001
12	unknown	2.27±0.49	0.65±0.3	0.005
13	platyphylloside	24.68±1.16	29.70±0.91	0.001
14	diarylheptanoid hexoside	71.09±6.22	ND	
15	centrolobol glycoside	8.15±0.94	3.92±0.78	<0.0001
16	centrolobol glycoside	16.44±0.82	24.15±1.29	<0.0001
17	centrolobol hexoside	14.86±0.69	14.08±1.10	0.523

P-values were generated by ANOVA (P<0.05)

[a]Contents expressed as mg per gram fresh weight (N=32±SE)

[b]*ND* Not detected

Table 3 Intraspecific variation in constitutive phenolics[a] present in stem phloem tissue of paper birch, *Betula papyrifera*, and European white birch, *Betula pendula*

		B. papyrifera				*B. pendula*			
No.	Compound	M1	M2	12	231	5818	5832	5938	50076
1	procyanidin dimer	6.5±0.4 a	6.0±0.4 a	6.9±0.9 a	7.9±0.6 a	6.6±0.6 a	6.7±0.4 a	6.9±0.2 a	6.0±0.2 a
2	catechin pentoside	**43.8±6.6 b**[b]	**88.1±9.7 a**	**53.2±7.3 b**	**63.4±9.2 ab**	113±11 a	107.9±8.1 a	109.3±0.2a	105.0±0.9 a
3	catechin	11.1±1.0 a	11.1±0.9 a	12.9±2.1 a	15.3±1.4 a	**13.0±1.3 b**	**13.0±0.9 b**	**16.0±0.7 a**	**11.7±0.5 b**
4	procyanidin trimer	**8.1±0.8 b**	**9.4±0.6 b**	**9.5±1.3 b**	**17.3±1.3 a**	**15.2±2.3 a**	**8.9±1.3 b**	**10.4±1.2 b**	**10.2±1.1 b**
5	coumaroylquinic acid	**4.7±0.6 a**	**1.9±0.5 b**	**3.6±0.3 a**	**3.4±0.6 a**	ND[c]	ND	ND	ND
6	betuloside pentoside A	10.5±2.0 a	11.9±0.9 a	10.6±2.2 a	16.4±3.8 a	ND	ND	ND	ND
7	betuloside	**18.2±1.7 b**	**23.6±2.1 b**	**23.7±6.8 b**	**52.4±8.0 a**	27.2±4.4 a	26.7±1.6 a	27.2±1.1 a	31.5±1.5 a
8	betuloside pentoside B	30.8±4.4 a	28.6±4.0 a	30.2±6.0 a	53.5±7.7 a	23.8±5.6 a	21.0±1.8 a	19.8±1.7 a	20.9±1.8 a
9	unknown	4.0±0.7 a	2.9±0.5 a	4.2±1.0 a	8.2±1.5 a	2.1±0.6 a	1.6±0.2 a	1.7±0.3 a	1.7±0.2 a
10	unknown	2.5±0.5 a	2.3±0.4 a	1.8±0.6 a	4.0±0.8 a	0.5±0.4 a	0.00±0.00 a	0.00±0.00 a	0.2±0.1 a
11	unknown	2.0±0.5 a	2.4±0.4 a	1.7±0.3 a	2.9±0.5 a	1.0±0.7 a	0.83±0.2 a	0.4±0.2 a	0.4±0.2 a
12	unknown	1.6±0.4 a	3.1±1.7 a	1.6±0.4 a	2.9±0.8 a	**1.7±0.9 a**	**0.00±0.00 b**	**0.00±0.00 b**	**1.0±0.2 ab**
13	platyphylloside	22.4±2.2 a	29.0±3.2 a	22.3±1.2 a	25.0±1.5 a	29.0±3.3 a	30.3±1.7 a	30.7±0.8 a	29.2±0.7 a
14	diarylheptanoid hexoside	77.2±9.8 a	65.5±9.1 a	61.6±11.1 a	81.4±20.2 a	ND	ND	ND	ND
15	centrolobol glycoside	6.9±1.9 a	11.5±0.9 a	4.0±1.5 a	10.5±2.0 a	**1.96±1.3 b**	**9.3±0.6 a**	**2.6±1.3 b**	**1.8±1.0 b**
16	centrolobol glycoside	16.7±1.9 a	18.4±1.6 a	15.0±0.9 a	15.6±2.0 a	**19.2±2.3 b**	**24.6±2.1 ab**	**22.5±1.6 b**	**30.3±2.9 a**
17	centrolobol hexoside	15.3±1.0 a	15.3±1.9 a	13.4±0.7 a	15.6±1.8 a	14.9±2.7 a	14.1±2.2 a	15.2±1.0 a	12.1±2.7 a

Means within a row followed by different letters are significantly different within each species by LSD ($P<0.05$)

[a] Contents expressed as mg per gram fresh weight ($N=8\pm$SE)

[b] Bold used to highlight significant differences among clones of each species

[c] *ND* Not detected

Discussion

We found important qualitative and quantitative differences between constitutive phenolics in stem phloem of paper birch, which is resistant to BBB, and European white birch, which is much more susceptible (Table 2, Fig. 3). Phloem tissues of paper birch contained coumaroylquinic acid, betuloside pentoside A, and a diarylheptanoid related to platyphylloside, which were not detected in European white birch and, therefore, may contribute to greater resistance of paper birch. Six compounds (betuloside pentoside B, a centrolobol glycoside, and four unknown compounds) found in higher concentrations in paper birch than in European white birch may also contribute to variation in resistance between species. Further research is needed to determine if any of these compounds contribute to interspecific variation in resistance to BBB by acting as toxins or antifeedents. Additionally, three compounds (catechin pentoside, platyphylloside, and a centrolobol glycoside) were found in higher concentrations in European white birch and might play a role as feeding or ovipostional stimulants, although we do not have any evidence that these chemicals modulate BBB behavior. Overall, paper birch and European white birch had similar phenolic profiles (Fig. 1), with 14 of 17 phenolics analyzed in this study present in both species (Table 1). This phytochemical similarity likely reflects their close phylogenetic relationship within the subgenus *Betula* (Schenk et al., 2008).

Concentrations of 24% and 36% of phenolics varied among clones and/or half-siblings of paper birch and European white birch, respectively (Table 3). However, there was no separation among clones and half-siblings within either species based on multivariate profiles of their phenolic composition (Fig. 3). In birch leaves and seedlings, intraspecific genetic variation in birch secondary metabolites is well documented (Keinanen et al., 1999; Laitinen et al., 2000, 2004; Ossipov et al., 2008) and is hypothesized to slow herbivore adaptation to a single chemical profile (Keinanen et al., 1999). In our study, the relative resistance of clones and half-siblings of paper birch and European white birch to BBB is not known. Therefore, it is difficult to make inferences on the potential role the variation (or lack thereof) in phenolic composition we observed has on BBB resistance. However a 12-year common garden study in Michigan, USA documented large intraspecific variation in resistance among half-siblings of paper birch, ranging from no colonization to high levels of colonization by BBB larvae (Miller et al., 1991). In contrast, there was little variation in resistance among half-siblings of European white birch, as all were highly susceptible to BBB colonzation (Miller et al., 1991).

Of the compounds for which some structural information could be established, four were monomeric/oligomeric flavanols (1–4), three were phenolic arylbutanoids (6–8), and five were phenolic diarylheptanoids (13–17) (Fig. 2). The monomeric flavanol catechin (3) and its stereoisomers are the building blocks of oligomeric procyanidins (condensed tannins) (1,4) (Hagerman and Butler, 1991), which we detected in birch phloem tissues. Diarylheptanoids (13–17), which possess anti-inflammatory, anti-hepatotoxic, and free radical scavenging activity, have been isolated from many plant genera including *Acer*, *Alnus*, *Betula*, *Curcuma*, *Juglans*, and *Zingiber* (Claeson et al., 1994; Akazawa et al., 2006). Platyphylloside (13) and centrolobol (15–17), which are glycosidic diarylheptanoids, also have been previously isolated from birch phloem (Smite et al., 1993; Julkunen-Tiitto et al., 1996). Both compounds have an inhibitory effect on ruminant digestion *in vitro* and are considered to be part of the chemical defense against mammalian herbivores (Sunnerheim et al., 1988; Sunnerheim and Bratt, 2004). Coumaroylquinic acids (5) are common intermediates in the phenylpropanoid biosynthetic pathway (Steck, 1968). All of these phenolic compounds have been detected previously either from stems or leaves of *Betula* spp. (Kolodziej, 1990; Smite et al., 1993; Julkunen-Tiitto et al., 1996; Mämmelä, 2001; Laitinen et al., 2004; Haviola et al., 2006; Karonen et al., 2006).

Previous research has suggested that birch species such as river birch (*B. nigra* L.), that lack rhododendrol (an arylbutanoid), are resistant to BBB (Santamour, 1990; Santamour and Lundgren, 1997; Nielsen et al., 2011). Moreover, Santamour (1990) found a short-range attraction of mated BBB females to this compound. Rhododendrol is generated by the hydrolysis of the glycosides containing this aglycone, including betuloside (also known as rhododendrin) and betuloside pentoside (Smite et al., 1993; Julkunen-Tiitto et al., 1996; Santamour and Lundgren, 1997). We found no difference among species in the levels of betuloside, and the highly resistant paper birch actually contained significantly higher levels of two betuloside pentosides than susceptible European white birch (Table 2). Therefore, although rhododendrol and/or its glycosides may mediate BBB oviposition behavior, their presence does not appear to contribute or relate to susceptibility.

The low molecular weight phenolics measured in this experiment are only one class of compounds that are present in birch phloem that could affect BBB larval performance. High molecular weight phenolics (tannins and lignins) (Wainhouse et al., 1990; Moilanen and Salminen, 2008), defensive proteins (Poyla, 2003; Dafoe et al., 2009), and nutritive components (amino acids, proteins, sugars, sterols, etc.) (Haack and Slansky, 1987; Slansky, 1992) are important mediators of other plant-insect interactions and could also impact growth and survival of wood-boring insects. Furthermore, while the goal of this study was to characterize constitutively expressed phe-

nolics, induced responses of birch to BBB are also likely important components of resistance and merit investigation.

In summary, we documented qualitative and quantitative differences in phenolic composition of stem phloem tissue between paper birch, which is resistant to bronze birch borer, and European white birch, which is more susceptible. Specifically, we found three compounds that were present only in paper birch and six other phenolics that were higher in concentration in paper birch compared to European white birch, which could contribute to the much higher resistance of paper birch. Developmental and behavioral bioassays are needed to determine if these differences contribute to the high resistance of paper birch to BBB. We also found genetic-based intraspecific variation in phenolic composition within both species, but clones and half-siblings within each species could not be distinguished by their phenolic profiles by using multivariate analysis.

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about the ecology of the Southern pouched lamprey, *Geotria australis*, which is endemic to New Zealand. Once an important traditional fishery, anecdotal evidence suggests that populations have declined steadily over time (James, 2008). Recent research has focused on enhancing knowledge of the distribution and abundance of *G. australis* populations within New Zealand, and developing methods for monitoring populations via pheromones released by stream resident larvae (Baker et al., 2009).

Within the laboratory, the larvae of *G. australis* produce large quantities of PS, with markedly lower amounts of PADS, and with PSDS undetected (Stewart and Baker, in press). Thus, population monitoring of *G. australis* is more likely to be successful by concentrating on detection of PS in river water. However, as a generally applicable method, it would be desirable to include PADS and PSDS in the methodology.

For monitoring of fish populations (using pheromone detection), a robust, rapid, and accurate measurement of these chemicals is essential. Liquid chromatography coupled to triple quadrupole mass spectrometry (LC/MS/MS) is ideally suited to this application, as it is highly sensitive and selective in the analysis of environmental concentrations of organic water soluble chemicals. As such, we explored the suitability of a pre-concentration step followed by LC/MS/MS analysis in quantifying PS, PADS, and PSDS in a river water matrix. Here, we describe the method process and validation.

Methods

Standards PS was purchased from Toronto Research Chemicals (P293526), while PADS and PSDS were kindly supplied by Professor Peter Sorensen, from the University of Minnesota.

Pre-concentration Lamprey pheromones were concentrated from the water matrix by Oasis HLB solid phase extraction (SPE) cartridges (Waters Corporation). SPE cartridges (60 mg) were conditioned with methanol (2 ml) and H_2O (2 ml), prior to sampling. Water samples (20 ml) were eluted through the SPE, using positive pressure. The water sample was eluted fully through the SPE cartridge and all eluent discarded. The cartridge then was removed of excess H_2O by several passes of air through with positive pressure. The material retained by the SPE cartridge was immediately eluted into a 2 ml amber glass vial with methanol (2 ml). All methanol was removed under a stream of N_2 gas. The sample was re-dissolved in 1:1 methanol:H_2O (100 μl), sonicated briefly, and transferred to a low volume 150 μl glass insert, inside the 2 ml amber glass vial. The sample was sealed with a lid and septum for LC/MS/MS analysis. All analyses were carried out in triplicate.

Spike Recovery PS, PADS, and PSDS were spiked into blank stream water at concentrations equivalent to 0.01 μg/l and 0.001 μg/l. Water from a stream with no known lamprey larvae (Donny Park Stream, a tributary of the Waikato River) was used for spiking and procedural blanks. Samples of stream water (100 ml) were spiked with each standard (100 μl at 1000-fold desired concentration), to keep methanol concentration negligible (0.1% v/v), and avoid compromising the performance of SPE.

Procedural Blanks Procedural blanks were used for stream water samples and were processed as above.

Post SPE Spikes To assess matrix interference procedural blank samples were spiked with standards (9.1 ng/ml) and analyzed by LC/MS/MS.

LC/MS/MS Analysis Quantitation of PS, PADS, and PSDS was achieved on an Agilent 6460 Triple Quadrupole mass spectrometer with an electrospray ionization (ESI) source coupled to an Agilent binary pump HPLC system (1200 Series Binary Pump SL, HiP-ALS SL+ autosampler and 1200 series degasser). Samples were analysed on an Agilent Zorbax 1.8 μm, 2.1×50 mm SB-C18LC column, using a gradient of mobile phase A (0.01 M ammonium acetate in Type 1 water) and mobile phase B (acetonitrile). Flow was 0.3 ml/min with an initial proportion of 20% B holding for 0.5 min, a linear gradient to 30% over 0.5 min, 60% over 2.5 min, 100% over 0.5 min, holding for 0.5 min, and back to 20% B over 0.5 min and holding for 1 min, a column temperature of 50°C was maintained. PS and PADS were analysed in positive ion mode, while PSDS was analysed in negative ion mode. PS was observed as the ammonium adduct at *m/z* 492.4 ($[M+H+NH_4]^+$) with specific transitions to *m/z* 359.3 for the quantifier ion (Quant) and *m/z* 412.4 for the qualifier ion (Qual). PADS was observed as the trihydrogen adduct ($[M+3H]^+$) at *m/z* 705.4, with the Quant ion at *m/z* 509.3 and the Qual ion at *m/z* 607.4. PSDS was observed as the doubly charged anion ($[M]^{2-}$) at *m/z* 294.3, with the Quant ion at *m/z* 491.3 and the Qual ion at *m/z* 97 ($[SO_4H]^-$).

Results

Quantification of Pheromones Spike recoveries for PS were 79.2 (2.5)% and 76.4 (8.1)% [(% Relative Standard Deviation (RSD)] for concentrations of 0.01 μg/l and

0.001 μg/l, respectively. Spike recoveries for PADS were 67.1 (5.5)% and 65.5 (3.0)% (% RSD) for concentrations of 0.01 μg/l and 0.001 μg/l, respectively. Due to poor fragmentation of PSDS in spike recoveries, it could only be quantified for the 0.01 μg/l spike experiments, with an average recovery of 141 (10)% (% RSD).

Post-spiking of procedural blanks showed a 92–95% response of the signal expected for all three pheromones, suggesting that a typical river matrix did not result in significant suppression or enhancement when using this method, at the higher 0.01 μg/l spike concentration.

The triple quadrupole analysis afforded instrument practical quantitation limits (PQL) of 0.05 μg/l for PS and PADS and 0.5 μg/l for PSDS.

Discussion

Previous methods for quantitation of lamprey migratory pheromones have been restricted to PS and allocholic acid (ACA) by using methods based around induced fluorescence by enzymatic oxidation of the 3α-hydroxy functional group (Polkinghorne et al., 2001), or direct infusion mass spectrometry by using selected ion monitoring (SIM) (Fine and Sorensen, 2005). The method of Polkinghorne et al. (2001) is time consuming (160 min), reasonably insensitive (detection threshold >0.01 μg in a clean background), requires a custom made column, is not selective to specific chemicals of interest (will detect any 3α-hydroxy bile acid), and will not detect PADS or PSDS. The method of Fine and Sorensen (2005) uses an ion trap mass spectrometer, which

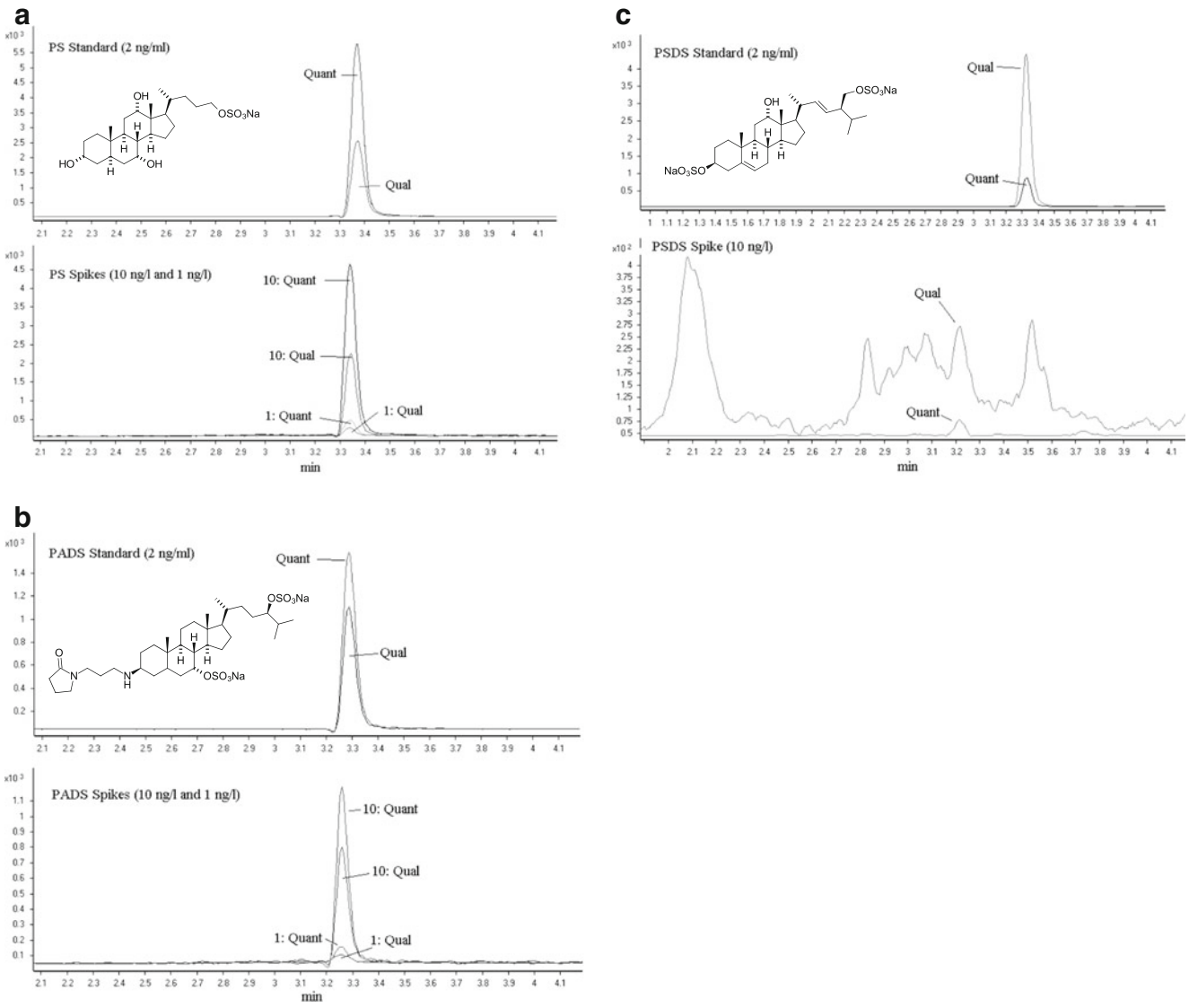


Fig. 1 LC/MS/MS chromatograms of PS, PADS and PSDS. **a** PS standard at 2 ng/ml (top trace) and spikes at 10 ng/l and 1 ng/l (bottom trace); **b** PADS standard at 2 ng/ml (top trace) and spikes at 10 ng/l and 1 ng/l (bottom trace); **c** PSDS standard at 2 ng/ml (top trace) and spike at 10 ng/l (bottom trace): Note different time scales for PSDS

is incapable of fragmenting PS and so relies on a narrow SIM window to detect and quantify this compound. Furthermore, with no chromatographic fractionation, matrix effects can be large and there is no retention time confirmation.

The LC/MS/MS method presented here has many benefits over existing methods for quantifying lamprey migratory pheromones. It uses a short highly efficient HPLC column to provide retention time confirmation of the compound of interest, and as such is extremely rapid, with a total run time of 7 min. It uses defined concurrent fragmentation of each compound of interest to form ions for quantitation (Quant) and ions to qualify (Qual) the existence of that compound (see Fig. 1). The method is further validated by analysis of Quant/Qual ion ratios and is extremely sensitive, with instrumental practical quantitation limits (PQL) of 0.05 mg/l for PS and PADS and 0.5 mg/l for PSDS. The PQL is defined as the minimum concentration of which quantification can be achieved with known level of accuracy and precision. A pre-concentration factor of 200 equates to "in water" PQL of 0.25 ng/l for PS and PADS and 2.5 ng/l for PSDS, corresponding to molar concentrations of 5×10^{-13} M, 7.5×10^{-13} M and 6.25×10^{-12} M for PS, PADS and PSDS, respectively. However, greater pre-concentration steps also are applicable, and a 4000-fold pre-concentration—as has been used in water sampling of streams (Stewart and Baker, 2011)—equates to "in water" molar PQLs of 2.5×10^{-14} M, 3.75×10^{-14} M, and 3.125×10^{-13} M for PS, PADS, and PSDS, respectively. The analysis is fully automated, is not labor intensive, and can process many samples in a short period of time. Furthermore, the suite of chemicals of interest can be expanded manifold without significantly increasing the method run time or impacting on other chemicals currently being analysed.

The analysis of PSDS by this method was not as robust as that of PS and PADS, with observed sensitivity reduced by 90%. This was largely a function of the poor fragmentation yield from the parent ion (*m/z* 294.3) to the quantifier ion (*m/z* 491.3) and low specificity of the relatively low molecular weight qualifier ion (*m/z* 97), leading to significant background noise from the matrix (Fig. 1c). Further avenues could be investigated to improve the detection limit of PSDS. However, this is the first described method for quantitation of PSDS and with suitable pre-concentration (4000×), sub picomolar quantitation can still be achieved.

A similar method for quantitation of the male sea lamprey sex pheromone, 3-keto-PS has been described recently (Xi et al., 2011). Like the current method, the method of Xi et al. uses triple quadrupole mass spectrometry for analysis. However, the authors noted that a mixed mode ion exchange cartridge was necessary to minimize matrix interference. Fragmentation of 3-keto-PS was not selective, with a daughter ion observed at *m/z* 97, as was observed for PSDS in the current method. Such a non-selective ion fragment likely accounts for the high matrix interference observed in both our method for PSDS and that of Xi et al. The method of Xi et al. uses negative ion mass spectrometry to quantify 3-keto-PS. We found that positive ion mass spectrometry—facilitated by ammonium acetate in the mobile phase—gave more useful fragmentation ions for PS and PADS than negative ion, leading to reduced matrix interference and associated lower PQL.

The methodology described in this paper is suitable for quantifying environmental concentrations of migratory lamprey pheromones. In addition, the methodology can be modified easily to measure other organic chemicals present in natural waters, such as hormones, pheromones, and metabolic by-products excreted by fish, provided suitable standards are available to achieve accurate quantitation. This method (or related methodologies) hopefully will provide a useful tool in species monitoring and management, and we aim to use this technique to develop an assay for assessing lamprey populations within New Zealand streams.

Acknowledgments The authors thank Professor Peter Sorensen from University of Minnesota for PADS and PSDS standards.

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Recently, we (Foster, 2009; Foster and Johnson, 2010) discovered that sugar feeding by adult female *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) increases pheromone and precursor titers, probably through increased glycolysis. This suggested an approach for quantifying pheromone biosynthesis at natural precursor levels by using ^{13}C-labeled sugar and MIDA. This system seemed suitable for MIDA because labeled sugar is rapidly absorbed into hemolymph (Foster, 2009) and then utilized at a steady rate for pheromone production (Wolfe and Chinkes, 2005).

In this report, we show, for the first time, application of MIDA to calculate synthetic rates of an insect-produced semiochemical.

Methods and Materials

Insects Insect rearing has been described elsewhere (Foster, 2009). Adult females (16:8 L:D), were collected daily and allowed to feed on water until used at 2 day. A 25 µl drop of glucose (either 99% U-$^{13}C_6$-glucose, Cambridge Isotope Laboratories, Andover MA, USA, or unlabeled) solution (10% w/w) was placed on a clean watch glass, and a virgin female allowed to feed. Only females that finished the drop (typically within 2 min) were analyzed. Timing started after completion of feeding. For the scotophase experiment (*Scot*), females fed during a window of 1 hr before to 3 hr into the scotophase. For the photophase experiment, females fed during the 2nd–5th photophase hours (*Phot*), and were sampled before the 9th photophase hour. These sampling times correspond to periods of high and low pheromone titers (see Results).

Pheromone Analyses Pheromone was analyzed by gas chromatography–mass spectrometry, as reported (Foster, 2009; see also, Supplementary). Only the major component, (*Z*)-11-hexadecenal (Z11-16:Ald), was subjected to titer analysis and MIDA, with *m/z* 220 (M-H_2O, a characteristic ion of unlabeled Z11-16:Ald, with carbon skeleton intact), and its two isotopomers (*m/z* 222 and 224, respectively), corresponding to incorporation of one or two $^{13}C_{1,2}$- acetyl CoA, recorded.

Results and Discussion

For *Scot* females, MIs of singly (*m/z* 222) and doubly labeled (*m/z* 224) Z11-16:Ald were detectable shortly after feeding, and rose over the first 4 hr (Fig. 1a). The tracer: tracee ratios of singly [TTR(M+1)] and doubly [TTR(M+2)] labeled Z11-16:Ald were calculated (Eqns 1 and 2, Supplementary), after correcting for abundance of naturally occurring isotopes and contribution of isotopic abundances of

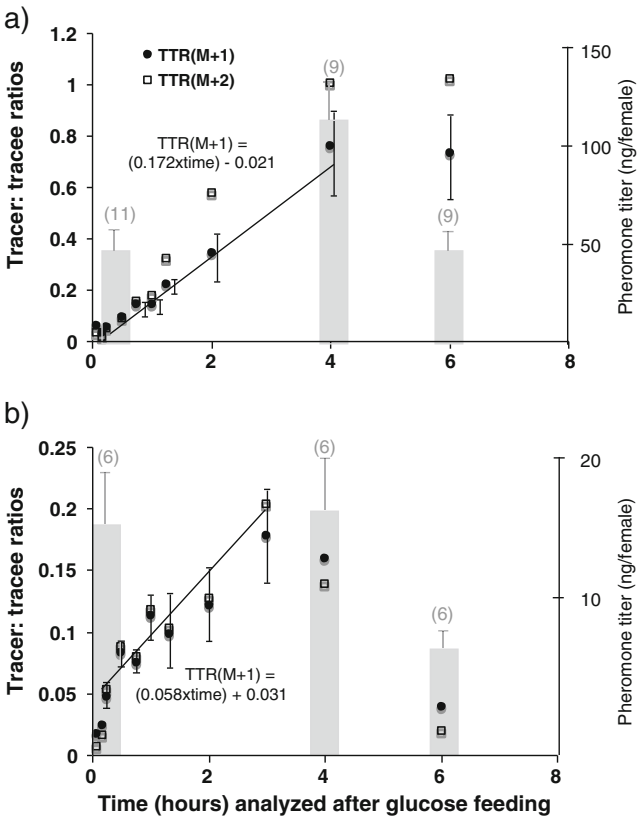


Fig. 1 Tracer to tracee ratios (TTRs) of corrected singly [TTR(M+1)] and doubly [TTR(M+2)] labeled (*Z*)-11-hexadecenal mass isotopomers, at various times after feeding on 25 µl of a 10% solution of U-$^{13}C_6$-glucose, for female *Heliothis virescens*. (**a**) Fed and analyzed during the scotophase; (**b**) fed and analyzed during the early photophase (3rd–9th hour). Mean pheromone titers (light grey bars) at various hours of the scotophase scotophase (0, 4 and 6 hr) and photophase (3, 7, and 9 hr) times are given in 1(**a**) and 1(**b**), respectively. SEMs are given for TTR(M+1)s and pheromone titers. Regression lines (and equations) for the rate of change of singly labeled (*Z*)-11-hexadecenal enrichment are shown. Sample sizes for determination of mean titers are given atop error bars. Each TTR point is the mean of 5–9 females

singly to doubly labeled MIs (Fig. 1). Acetate p (Eqn 3, Supplementary) was calculated as 0.315, and converted to units of singly labeled Z11-16:Ald enrichment (Ep) (Eqn 4, Supplementary), yielding a value of 0.178. The rate of increase of singly labeled Z11-16:Ald enrichment before isotopic steady state (i.e., the plateau) was calculated by regression as 0.172/h (Fig. 1a). Dividing this by Ep (Eqn 5) yielded a fractional synthetic rate (FSR) of 0.965; i.e., 95.5% of the net Z11-16:Ald pool per hour is synthesized from acetate derived from glucose. Crudely averaging (using three times; Fig. 1a) Z11-16:Ald titer over the scotophase gave a value of 66.1 ng, yielding an average absolute synthetic rate (ASR) of 63.1 ng/h.

The *Phot* TTRs of Z11-16:Ald MIs showed a steady increase until roughly 2–4 hr after feeding, and then declined (Fig. 1b). This decline later in the photophase

suggests that at this time pheromone was increasingly biosynthesized from pools of acetate other than those generated from the ingested sugar (i.e., in hemolymph trehalose), most likely from recycled (unlabeled) fatty acids (Foster, 2005). Decreased biosynthesis of pheromone from hemolymph trehalose at this later part of the photophase was supported by the lower mean titer at the 9th hour of the scotophase compared to the two earlier times. Using similar calculations to that for the *Scot* data, photophase values of 0.237 and 0.285 of p and Ep, respectively, were calculated. Singly labeled Z11-16:Ald enrichment increased at 0.058/h (Fig. 1b), allowing calculation of the FSR during the first half of the photophase of 0.204 (i.e., 20.4% of the Z11-16:Ald pool per hour is synthesized from glucose). Crudely averaging (using the two times; Fig. 1b) the Z11-16:Ald pool during this period gave a mean of 15.8 ng, which gives an ASR of 3.2 ng/h.

These data show very large differences in synthetic rates of pheromone derived from hemolymph sugar between the two photoperiodic times. During the peak period of synthesis (*Scot*), when pheromone is released, ASR is nearly 20 times that during the photophase when pheromone is not being released. This difference presumably is due to the presence/absence of PBAN in the hemolymph, as it is consistent with differences in amounts of *de novo*-synthesized pheromone during *in vitro* assays using the presence and absence of PBAN (Jurenka et al., 1991; Rafaeli and Jurenka, 2003).

The high synthetic rate of pheromone throughout the scotophase means that roughly 430 ng (8×53.9; 1.8 nmoles) of pheromone is produced in this period. Assuming a total hemolymph volume of 50 µl (Raina et al., 1994), at a trehalose concentration of roughly 75 mM (Foster and Johnson, 2010), then there is roughly 3.75 µmoles of trehalose (= 7.5 µmoles glucose = 15 µmoles acetyl CoA) potentially available. Since eight molecules of acetyl CoA make one pheromone molecule, then the amount of pheromone produced during a single scotophase represents roughly 0.1% of potential precursor from hemolymph trehalose. In reality, more precursor will be expended, since large amounts of fatty acids are also synthesized (Foster, 2005). The high FSR (95.5%), along with the fairly constant titer throughout this period, indicates that not only is pheromone being turned over very rapidly in the gland, but that pheromone production is roughly in steady state with pheromone release and degradation.

Typically, it can be problematic in MIDA experiments obtaining values of TTR(M+2) large enough to be accurately measured (Wolfe and Chinkes, 2005). However, we obtained relatively large TTR(M+2) values, even shortly after feeding. This indicates that ingestion and absorption of labeled glucose occur rapidly and at a high level, giving high precursor enrichment in trehalose and pheromone gland acetate precursor pools, in female moths. Thus, this system seems especially suited to MIDA.

The application of MIDA to determine pheromone biosynthetic rates in *H. virescens* should facilitate determination of more quantitative measures of pheromone production, under different physiological and environmental conditions, in this and other moths. Moreover, this approach has wider applicability to studying synthetic rates of semiochemicals derived from glycolysis in other sugar-feeding insects.

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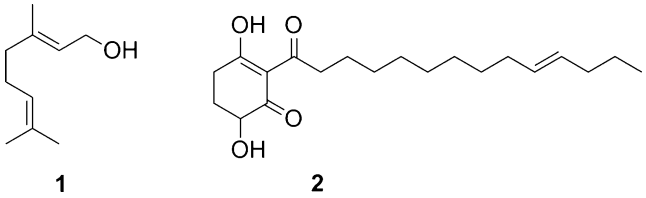


Fig. 1 Chemical structures of geraniol (1) and 3,6-dihydroxy-2-[1-oxo-10(*E*)-tetradecenyl]-cyclohex-2-en-1-one (2)

Although an alarm pheromone response has been described in *C. ciliata* (Kearns and Yamamoto, 1981), there has been no further exploration of the chemical compounds involved, hence the initiation of this alarm pheromone study.

Methods and Materials

Study Species Sycamore lace bugs, *Corythucha ciliata* (Say), were collected from the London Plane, *Platanus* x *hispanica,* at Kyoto Gakuen University in 2009, and were used for all chemical and behavioral studies in the laboratory without any preliminary conditioning.

Chemical Analysis Gas chromatography-mass spectrometry (GC-MS) analysis was conducted using a Network GC System (6890 N; Agilent Technologies Inc.) coupled with a mass selective detector (5975 Inert XL; Agilent Technologies Inc.) operated at 70 eV, using an HP-5MS capillary column (0.25 mm i.d. × 30 m, 0.25 μm film thickness; Agilent Technologies Inc.). Helium was used as carrier gas at a flow rate of 1.00 ml/min, with a split-less mode at a temperature programmed to change from 60°C (2 min) to 290°C at a rate of 10°C/min. This 290°C temperature was then maintained for 5 min. GC and GC-MS data were obtained by Chemstation (Hewlett Packard Co.), with reference to an MS database (Wiley275 library; Hewlett Packard Co.).

Mass Extraction Nymphs (ca. 1 ml volume) of all developmental stages were collected from leaves by brushing. They were dipped subsequently in hexane (3 ml) for 3 min, and the resulting extracts were obtained by decantation. The residue was washed twice with hexane (3 ml), and the combined extracts, after concentration *in vacuo*, were subjected to a SiO_2 column chromatography to purify the alarm pheromone.

Individual Extraction Individual adults and nymphs at specific developmental stages were each placed separately into a glass tube (0.1 ml volume with a conical bottom, glass vial insert, Agilent Technologies Inc.). Potential alarm pheromones were extracted with hexane (4 μl). After 3 min, the extract was collected with a 10 μl micro-syringe, and subjected to GC–MS analysis or bioassay for alarm pheromone activity. For quantitative analyses, we extracted groups of five individuals at each developmental stage with hexane (4 μl, five replications per stage). Extracts then were subjected to GC–MS analysis.

Bioassay Method Escape behavior was observed at 25°C with ambient humidity, using a leaf bearing a colony (ca. 1 cm diam) with ca. 100 nymphs (3 rd-5th instars) on its underside. The leaf was held upside down, and a piece of filter paper (1×2 mm), with a known impregnated amount (ng/μl) of test compound or volume of extract, was placed at the center of the aggregation with minimum disturbances.

We defined a filter paper sample to be active or inactive according to the strength of avoidance behavior exhibited by nymphs moving away from the filter paper. Avoidance behavior was measured (N=5 trials) as the time in seconds for all nymphs to reach more than 1 cm from the test paper. When all nymphs took less than 2 min to reach more than 1 cm distance, the dose and compound were defined as active. Doses and compounds that resulted in avoidance behavior slower than 2 min were defined as inactive. Time data were analyzed using ANOVA; *post hoc* comparisons were made with Tukey's test.

Determination of Geraniol Content Authentic geraniol (**1**, reagent grade, Wako Pure Chemical Industries, Ltd.) was diluted with hexane. The following concentrations were prepared: 0.1, 0.5, and 1 ppm. A dose–response curve was obtained by correlating GC–MS response data with each volume (1 μl=1 ng) of three standard solutions (N=4 trials).

Results and Discussion

All developmental stages of *C. ciliata* are found on the underside of London Plane *P.* x *hispanica* leaves, and nymphs aggregate to form colonies varying in sizes from 0.5 cm to ca. 3 cm diam. When a crushed nymph is introduced into the center of a colony, nearby nymphs show evasive behavior (Kearns and Yamamoto 1981). In our study, all nymphs within 1 cm dispersed within 2 min. This phenomenon was reproduced when a filter paper impregnated with one nymph-equivalent of extract was introduced to a colony. In comparison, nymph avoidance behavior was not observed in response to adult extracts. The active component occurred only in nymphs, and functions as an alarm pheromone for other conspecific nymphs.

Hexane extracts from a 5th-stage nymph and an adult were compared by GC–MS analyses (Fig. 2). A small but distinctive compound (**1**) at t_R 10.013 min was detected

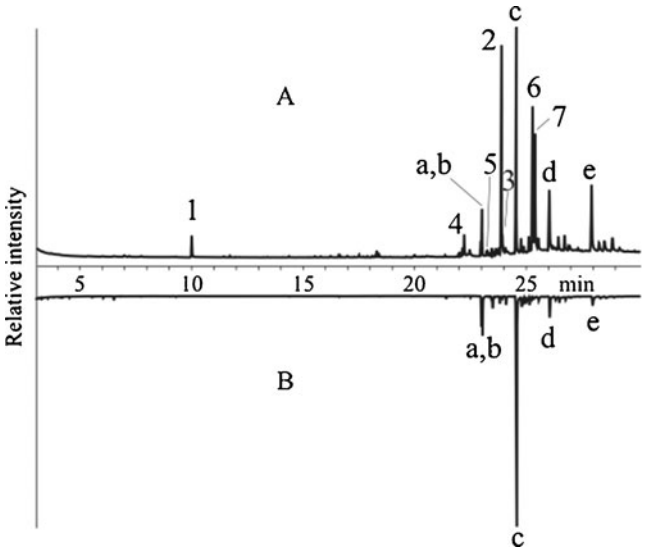


Fig. 2 Gas chromatographic comparison of hexane extracts from a nymph and an adult. A, Fifth nymphal stage; B, adult. **1**, geraniol; **2**, 3,6-dihydroxy-2-[1-oxo-10(*E*)-tetradecenyl]-cyclohex-2-en-1-one; **3**, 3,6-dihydroxy-2-[1-oxo-tetradecyl]-cyclohex-2-en-1-one; **4**, 3,6-dihydroxy-2-[1-oxo-dodecyl]-cyclohex-2-en-1-one; **5**, 3-hydroxy-2-[1-oxo-tetradecenyl]-cyclohex-2-en-1-one; **6**, 3,6-dihydroxy-2-[1-oxo-hexadecadienyl]-cyclohex-2-en-1-one; and **7**, the structure unknown with M^+ ion at *m/z* 364 and the base ion at *m/z* 139. **a**, *n*-docosanol; **b**, *n*-pentacosane; **c**, *n*-heptacosane; **d**, *n*-nonacosane; and **e**, *n*-hentriacontane

from extracts of the nymph, as well as those of other stages (data described below). In contrast, no trace of the corresponding peak was found in extracts of adult. Compound **1** indicated M^+ ion at *m/z* 154(2%) and the base ion at *m/z* 69(100%) with the diagnostic fragment ions *m/z* 139(4%), 136(4%), 123(13%), 121(8%), 111(7%), 93 (29%), 84(8%), 55(8), 53(9%), and 41(53%), suggesting an open chain monoterpene. The authentic compound **1** showed its GC peak at t_R 10.008 min, and gave an identical mass spectrum to those obtained from nymphal extracts. Hence, the structure of compound **1** was identified as *E*-3,7-dimethyl-2,6-octadien-1-ol, geraniol (**1**) (Fig. 1).

After evaporation of the solvent *in vacuo*, hexane extracts were applied to a SiO_2 (200 mg, Wako-gel C200; Wako Pure Chemical Industries, Ltd.) column and eluted stepwise with a 2-ml mixture of hexane, then 5, 10, and 50% ethyl acetate in hexane. Activity was found in the fraction eluted with 50% ethyl acetate in hexane. The active fraction was purified further on a SiO_2 (1 g) column eluted with a mixture of 50% ethyl acetate in hexane. Then, the eluate was collected into five fractions (each 1 ml), and the alarm pheromone activity was detected in the third fraction. Active fractions in each column chromatography contained **1** commonly as their major component. Except for compound **1**, no other compounds such as (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, linalool, or nerolidol aldehyde were detected in *C. ciliata*, as reported by Kearns and Yamamoto (1981). We, therefore, concluded the alarm pheromone to be compound **1**.

Nymphs were separated into five groups (designated as 1st, 2nd, 3rd, 4th, and 5th developmental stages, respectively) by their body sizes. The content of compound **1** in each respective developmental stage was 0.29±0.04 ng, 0.54±0.07 ng, 1.16±0.48 ng, 2.30±0.55 ng, and 4.20±1.50 ng (N=5 for each developmental stage) (Fig. 3). Compound **1** showed the following activity: 162.2±35.7 s (N=5) at 0.5 ng, 90.6±26.3 s (N=5) at 1 ng, and 42.4±11.9 s (N=5) at 3 ng (Fig. 4). Compound **1** was concluded to be active at a dose of 1 ng. Under the same conditions, one individual 5th-instar nymph took 45.7±13.1 s (N=5) to move 1 cm from filter paper impregnated with compound **1** (Fig. 4). Since compound **1** elicited activity at a dose of more than 1 ng, and a nymph older than the 3rd stage contained more than 1 ng of the compound, the alarm pheromone of *C. ciliata* was identified as compound **1**.

Kearns and Yamamoto (1981) first reported the presence of alarm pheromones in *C. cydoniae*, *C. marmorata, C. ciliata*, and *G. solani* by observing each of their alarm behaviors in response, not only to conspecific nymphs, but also to the other tingid species. Aldrich et al. (1991) identified acetaldehyde, *E*-2-hexenal, *E*-4-oxo-2-hexenal, **1**, and nerolidol aldehyde from anterior and posterior dorsal abdominal glands of *G. solani,* and a similar set of compounds (acetaldehyde, *E*-2-hexenal, **1**, linalool, and nerolidol aldehyde) from *C. cydoniae.* They proposed that the five compounds listed above, excluding nerolidol aldehyde, are involved in their alarm messages, although the alarm pheromone of *C. marmorata* and *C. ciliata* remains inconclusive.

In our study, the alarm pheromone of *C. ciliata* was identified as **1**; none of the other components described above (except acetaldehyde, not detectable by the present GC-MS condition) was recognized in the active SiO_2 fraction, and the dose–response of **1** alone was confirmed as comparable to that of nymph extracts. Cross activity

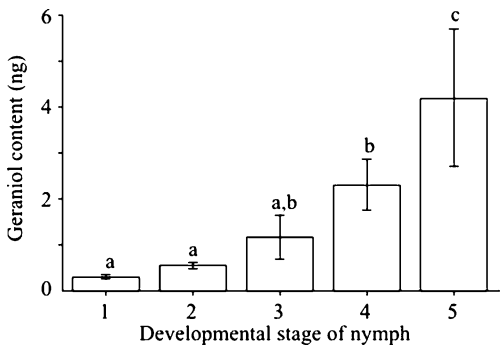


Fig. 3 Geraniol content in nymphal stages. Means capped with the same letter are not significantly different by Tukey's HSD test (P<0.05). Bars represent the mean±S.D. (N=5)

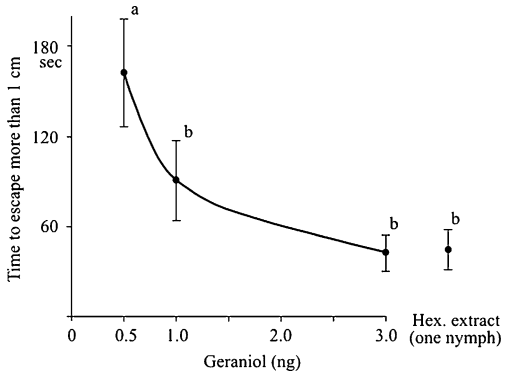


Fig. 4 Alarm pheromone activity of geraniol (**1**), and of hexane extract from a fifth-instar nymph. Means capped with the same letter are not significantly different by Tukey's HSD test ($P<0.05$). Bars represent the mean±S.D. ($N=5$)

among lace bugs may be explained by the presence of **1**, which is a compound common to all lace bugs analyzed (Kearns and Yamamoto 1981).

The natural chemical, 3,6-dihydroxy-2-[1-oxo-10(*E*)-tetradecenyl]-cyclohex-2-en-1-one [**2**, Figs. 1 and 2, M^+ ion at *m/z* 336(72%), the base ion at *m/z* 55(100%) and characteristic third-high-intensity ion $(C_9H_{11}O_4)^+$ at *m/z* 183(43%)] were reported from nymph stages of sycamore lace bug along with other related five minor compounds whose M^+ ions are only mentioned as *m/z* 338, 336, 362, 346, and 362, respectively (Lusby et al., 1987), however their structures remain obscure. In the present study, compound **2** (Fig. 1) was detected as peak **2** at t_R 23.776 min with M^+ and the base ion at *m/z* 336(100%) and the second highest intensity ion at *m/z* 183(50%).

In addition to peak **2,** five other compounds [peaks **3**–**7**, among which M^+ ions of peaks **5**–**7** corresponded to those reported (Lusby et al., 1987)] also were observed in the GC chromatogram as specific nymphal components. The MS libraries, analyses of mass spectra and co-chromatography to those of *Plodia interpunctella* larva (modified from Kuwahara et al., 1983, and extracted from a larva by dipping in hexane for 3 min.) were combined to elucidate the structure of these compounds.

Compound **3** was concluded to be 3,6-dihydroxy-2-[1-oxo-tetradecyl]-cyclohex−2-en-1-one, because it appeared as a shoulder peak at t_R 23.839 min with M^+ ion at *m/z* 338 (M^+ ion, 79%, two mass unit larger than that of compound **2**) and its base ion at *m/z* 183 (100%), indicative of saturated 2-(1-oxo-alkyl) analogue of compound **2**. The spectrum also was identical to MS libraries. Compound **4** [t_R 22.166 min with M^+ ion at *m/z* 310(57%) and the base ion at *m/z* 183(100%)] was concluded to be 3,6-dihydroxy−2-[1-oxo-dodecyl]-cyclohex-2-en-1-one, based on the fact that the same mass spectrum was demonstrated in *P. interpunctella* extract at t_R 22.155 min, in addition to MS libraries. The structure of compound **5** [t_R 23.145 min with M^+ ion at *m/z* 320(5%) and the base ion at *m/z* 167(100%)] was elucidated to be 3-hydroxy-2-[1-oxo-tetradecenyl]-cyclohex-2-en-1-one, comparing the mass spectrum of an homologue, 3-hydroxy-2-[1-oxo-hexadecyl]-cyclohex-2-en-1-one [the same fragmentation pattern with the same base ion (*m/z* 167, 100%), but different M^+ ion (*m/z* 350=*m/z* 320+28 +2, 10%)] present in *P. interpunctella* (Kuwahara et al., 1983) at t_R 24.808 min by co-chromatography. Mass spectrum of compound **6** at t_R 25.168 min with M^+ ion at *m/z* 362(32%) and the base ion at *m/z* 183 (100%) was similar to those of compounds **2** and **5**, and the structure of compounds **6** was elucidated as 2-(1-oxo-alkadienyl) homologue, 3,6-dihydroxy-2-[1-oxo-hexadecadienyl]-cyclohex-2-en-1-one. The MS libraries were not available. Peak **7** at t_R 25.290 min, seems to correspond to *m/z* 346 with one exchangeable hydrogen (Lusby et al., 1987), but its structure still remains obscure with the following mass spectrum [*m/z*(% rel.int.)]; 364(M^+, 0.9), 346(0.8), 328 (61), 300(2.6), 261(5.3), 243(12), 229(18), 221(37), 203 (36), 189(69), 177(17), 167(61), 154(63), 139(100), 126 (17), 111(20), 95(19), 79(34), 67(55), 55(40) and 41(22). These compounds (peaks **1**–**7**) were not detected in extracts of adults. Sexual dimorphism of components also was not observed using GC–MS in hexane extracts (data not indicated).

Other compounds (**a** - **e**) were equally distributed between nymph (A) and adults (B) in Fig. 2. Compound **a** at t_R 22.918 min was identified as *n*-docosanol. Its mass spectrum [*m/z*(% rel.int.)] was as follows; 308 (M^+-H_2O) (3.5), 280(3.9), 264(0.4), 252(0.8), 238(1.2), 222(1.4), 208 (1.6), 196(2.0), 182(2.8), 167(4.3), 153(4.7), 139(6.2), 125 (29), 111(75), 97(99), 83(100), 69(81), 57(83) and 43(64), and was identical to that of the authentic *n*-docosanol at t_R 22.927 min. The other compounds (**b** - **e**) were each identified as *n*-pentacosane (M^+ ion at *m/z* 352, peak **b**), *n*-heptacosane (M^+ ion at *m/z* 380, peak **c**), *n*-nonacosane (M^+ ion at *m/z* 408, peak **d**) and *n*-hentriacontane (M^+ ion at *m/z* 436, peak **e**), based on their characteristic sigmoid decreases of fragment ion intensities from *m/z* 57 to each *m/z* (M^+-29) fragments, respectively.

2-Acylcyclohexan-1,3-diones [3-hydroxy-2-(1-oxo-alkyl)-cyclohex-2-en-1-one] and 4-hydroxy-2-acylcyclohexan-1,3-diones [3,6-dihydroxy-2-(1-oxo-alkyl)-cyclohex-2-en-1-one], whose saturated-, monoene-, and diene-acyl moieties are composed mainly of C_{16}, C_{18}, and C_{20} carbon chains, are known as epideictic pheromones in larvae of four phycitid moths (the almond moth *Cadra cautella*, the Indian meal moth *P. interpunctella*, the Mediterranean flour moth *Anagasta kuehniella*, and the tobacco moth *Ephestia elutella* (Corbet, 1971)), and as an oviposition pheromone (Corbet, 1973). These compounds additionally exploited as a kairomone of the parasitic wasp *Venturia canescens*

(Corbet, 1971; Mudd and Corbet, 1973). The epideictic pheromones present in each phycitid appear to be species-specific, although compositions of 2-acylcyclohexan-1,3-diones are comparable among the four species (Kuwahara et al., 1983; Nemoto et al., 1987a, b). Although biological functions of these compounds remain unknown in the sycamore lace bug, it is nevertheless interesting to note that two taxonomically independent insect species share the same groups of compounds.

Compound **1**, distributed widely among plants as a minor component of their essential oils, also is found in astigmatid mites as a biosynthetic precursor of geranial and possibly of neral, and functions either as an alarm, aggregation, or sex pheromone (Kuwahara, 2004). Its geraniol dehydrogenase has been purified and characterized phylogenetically as a new class of enzyme (Noge et al., 2008). The honeybee *Apis mellifera* contains this compound as a component of the Nasanov gland pheromone (Free et al., 1981), and in the pepper weevil *Anthonomus eugenii* it functions as an aggregation pheromone (Eller et al., 1994).

Compound **1** is a stable monoterpene with agreeable flavor so it may be useful as a in conjunction with insecticide for lace bug control. As well as functioning as an alarm pheromone, compound **1** may irritate and force *C. ciliata* individuals to move around such that they more frequently come into contact with applied insecticides. This may improve the effectiveness of insecticides for lace bug control, and reduce environmental hazards caused by insecticide toxins.

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Methods and Materials

Insects Larvae of *S. salinella* were collected from glasswort at Gimje (35.4°N, 126.4°E), Korea in early May 2010. Larvae were reared on glasswort in screen cages (30×30×30 cm), and maintained at 25°C under an L14:D10 photoperiod. Pupae were separated by sex and kept individually in plastic bottles (7 cm high and 2.5 cm diam). After eclosion, moths were provided with a cotton pad soaked with a 10% sucrose solution as food.

Chemicals Synthetic *E*3-12:OAc, *Z*3-12:OAc, *E*5-12:OAc, *Z*5-12:OAc, *E*5-14:OAc, and *Z*5-14:OAc were purchased from Pherobank (Wageningen, The Netherlands). Isomeric purity of these compounds exceeded 99%. Dimethyldisulfide, iodine, and sodium thiosulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Pheromone Extraction Preliminary observations revealed that female *S. salinella* exhibited calling behavior during the second half of the scotophase. Therefore, the abdominal tips with pheromone gland of 1- to 2-d-old females were removed 7.0-7.5 hr into scotophase, and placed in 10 μl of hexane in a 0.3-ml conical glass vial (Wheaton, Millville, NJ, USA) for 30 min. The supernatant was transferred into another vial and stored at −20°C until gas chromatographic-mass spectrometry (GC-MS) analysis.

Dimethyldisulfide Derivatizations Double bond positions in unsaturated compounds were determined from dimethyl-disulfide (DMDS) adducts of the female-produced compounds (Buser et al., 1983). DMDS derivatization of a 47-female gland extract was performed as previously described by Yang et al. (2008).

Collection of Female Emissions Solid phase microextraction (SPME) was used to collect pheromone compounds emitted by calling *S. salinella* females. A SPME fiber (100 μm polydimethylsiloxane coating, Supelco, Bellefonte, PA, USA) was conditioned before use at 250°C for 30 min in a GC injector. Three virgin females were placed overnight at room temperature into a 10 ml glass vial containing a SPME fiber. Then the SPME fiber was desorbed for 30 sec in the injection port to start the GC-MS analysis.

Chemical Analysis Gland extracts and SPME-collected volatiles were analyzed on an Agilent 6890N GC interfaced to an Agilent 5975C mass-selective detector. Samples were run on DB-Wax and DB-23 columns (30 m×0.25 mm ID, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). Helium was the carrier gas (1 ml/min), and the injections were splitless. Injector temperature was 250°C, and the GC oven temperature was programmed from 80°C (1-min hold) to 200°C at 5°C/min and held for 10 min. The ionization voltage was 70 eV, and the ion source temperature was 230°C. Compounds were identified by comparison of Kováts retention indices (KIs; Marques et al., 2000), and mass spectra with those of authentic standards on two different columns. DMDS-derivatized gland extract was analyzed with a DB-23 column, using a temperature program of 100°C to 240°C at 10°C/min with other GC conditions as those described above.

Field Experiments Field trials were conducted in June of 2010 in heavily infested glasswort fields at Gimje, Korea. Sticky delta traps (Green Agro Tech, Gyeongsan, Korea) were hung from sticks at a height of 50 cm. Traps were baited with white rubber septa (8 mm OD; Aldrich Chemical, Milwaukee, WI, USA) loaded with test compounds in hexane. Two 1- to 2-d-old virgin females in small mesh cages were used as positive controls. All field tests employed a complete randomized block design with four replicate blocks. The distance between traps within a block was 10 m, and the distance between blocks was approximately 150 m.

Experiment 1 investigated the attraction of *S. salinella* males to *Z*3-12:OAc, *E*3-12:OAc, *Z*5-12:OAc, and *Z*5-14:OAc singly and in binary blends. Experiment 2 tested *Z*3-12:OAc in binary, ternary, and quaternary combinations with *E*3-12:OAc, *Z*5-12:OAc, and *Z*5-14:OAc. Finally, experiment 3 was conducted to determine the effects of addition of *Z*5-12:OAc at different doses to 100 μg of *Z*3-12:OAc. Trap catch data (x) were transformed to log (x + 1) to normalize the variance before analysis. Means were compared by one-way analysis of variance (ANOVA) followed by Tukey's test at α=0.05 (SAS Institute Inc., 2008).

Results

Chemical Analysis GC-MS analysis of pheromone gland extracts of *S. salinella* females revealed one major component and three minor components along with minor unknown compounds (Fig. 1). The mass spectrum of compounds 1, 2, and 3 were similar and had diagnostic fragment ions *m/z* 166 (M^+-CH_3COOH), 138, 124, and 61 ($CH_3COOH_2^+$), suggesting dodecenyl acetates. The DMDS adduct of compounds 1 and 2 showed a molecular ion *m/z* 320 and diagnostic ions *m/z* 173 ($C_9H_{18}SCH_3^+$) and 147 ($O_2C_5H_8SCH_3^+$), proving the addition of DMDS to a double bond at position 3 of dodecenyl acetates (Buser et al., 1983). Comparison of KIs of the natural compounds with *E*3-12:OAc and *Z*3-12:OAc standards on the DB-Wax

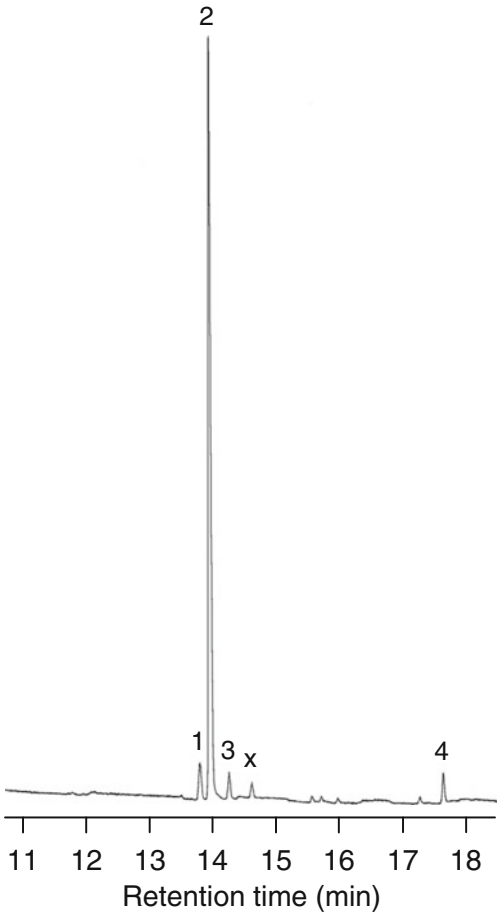


Fig. 1 Total ion chromatogram (TIC) of GC-MS analysis of pheromone gland extract from female *Scrobipalpa salinella* on a DB-23 column. Compound identities as follows: 1) *E*3-12:OAc, 2) *Z*3-12:OAc, 3) *Z*5-12:OAc, x) unknown, 4) *Z*5-14:OAc

and DB-23 columns confirmed that compounds 1 and 2 were *E*3-12:OAc (KIs of 1913 and 1917, respectively) and *Z*3-12:OAc (KIs of 1917 and 1927, respectively). The mass spectrum of the DMDS adduct of compound 3 gave diagnostic fragments at *m/z* 320 (M^+), 145 ($C_7H_{14}SCH_3^+$), and 175 ($O_2C_7H_{12}SCH_3^+$), indicating the original double bond at position 5 of the monounsaturated acetate. Although *E*5-12:OAc and *Z*5-12:OAc standards were not separated on the DB-Wax column, we confirmed compound 3 as *Z*5-12:OAc by matching KI to that of standard on the DB-23 column (KI=1944; KI of *E*5-12:OAc was 1934).

Compound 4 showed diagnostic ions *m/z* 194 (M^+-CH_3COOH), 166, 138, 124, and 61 suggestive of a tetradecenyl acetate. The mass spectrum of a DMDS adduct with M^+ at *m/z* 348, derived from the tetradecenyl acetate in gland extract, showed 173 ($C_9H_{18}SCH_3^+$) and 175 ($O_2C_7H_{12}SCH_3^+$), placing the double bond at position 5. KIs of compound 4 on the DB-Wax and DB-23 columns were identical to those of the *Z*5-14:OAc (KIs of 2125 and 2155, respectively) standard rather than *E*5-14:OAc (KIs of 2128 and 2146, respectively). The amount (±SE) of the major component *Z*3-12:OAc present in the gland extract was estimated as 1.2± 0.2 ng per female (*N*=12), the relative ratio of *E*3-12:OAc, *Z*3-12:OAc, *Z*5-12:OAc, and *Z*5-14:OAc was 8:100:5:7.

GC-MS analysis of the SPME samples from live females revealed the presence of *Z*3-12:OAc and *Z*5-12:OAc by their GC retention times and mass spectra in comparison with those of authentic standards (Fig. 2). Although there was considerable variability among samples, the mean ratio (±SE) of *Z*3-12:OAc and *Z*5-12:OAc was estimated to be 100:54±31 (*N*=3). In contrast, *E*3-12:OAc and *Z*5-14:OAc identified from gland extracts were not found in emissions of females.

Field Experiments A total of 2,755 *S. salinella* male moths were caught in field experiment 1. Traps with *Z*3-12:OAc, *E*3-12:OAc, *Z*5-12:OAc, or *Z*5-14:OAc as single components or blend containing *Z*3-12:OAc plus *Z*5-14:OAc caught few moths (Fig. 3). In contrast, traps baited with *Z*3-12:OAc together with either *E*3-12:OAc or *Z*5-12:OAc caught significantly more males than did traps baited with a hexane control. The binary blend of *Z*3-12:OAc and *Z*5-12:OAc captured approximately five times more males than caged females, while the blend containing *Z*3-12:OAc and *E*3-12:OAc caught similar numbers of moths as the virgin females. In field experiment 2, a total of 5,370 moths were

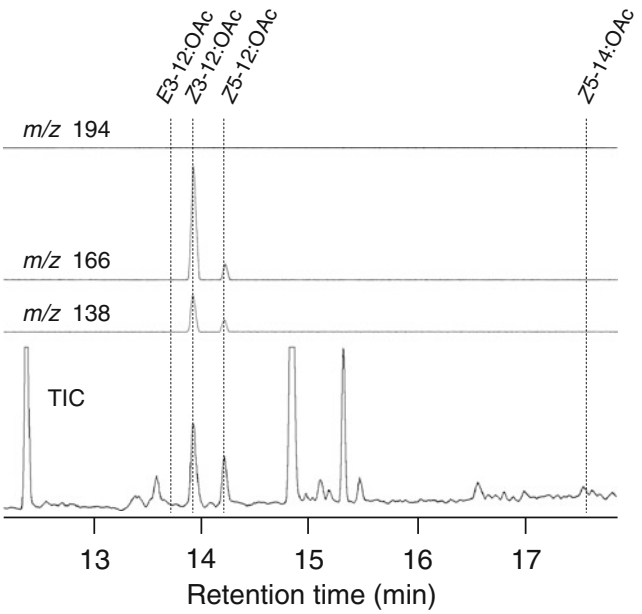


Fig. 2 TIC and the selected ion chromatogram of SPME samples from female *Scrobipalpa salinella*. Selected ions were monitored at *m/z* 194, 166, and 138 for tetradecenyl acetates, and *m/z* 166 and 138 for dodecenyl acetates

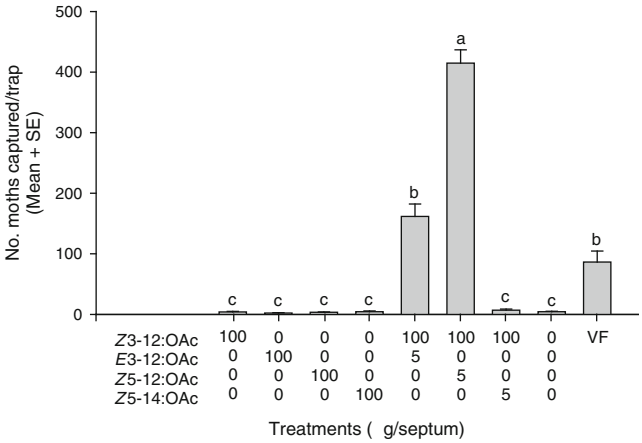


Fig. 3 Catches of male *Scrobipalpa salinella* in traps baited with *Z*3-12:OAc, *E*3-12:OAc, *Z*5-12:OAc, and *Z*5-14:OAc singly and in binary blends in glasswort fields in Gimje, Korea, 14–16 June 2010. Bars with the same letter are not significantly different (Tukey's test: $P>0.05$). VF = two virgin females

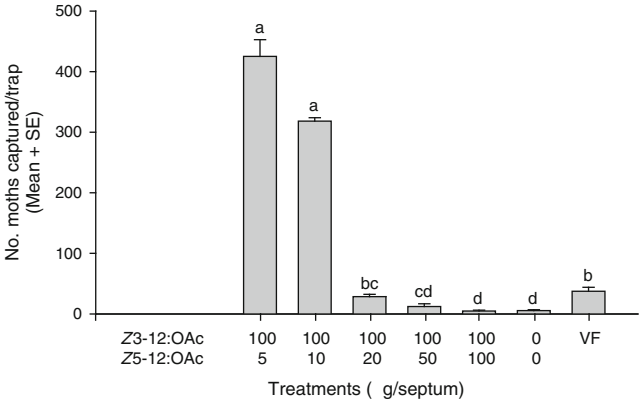


Fig. 5 Effect of addition of *Z*5-12:OAc at different doses to 100 μg of *Z*3-12:OAc on captures of male *Scrobipalpa salinella* in glasswort fields in Gimje, Korea, 18–20 June 2010. Bars with the same letter are not significantly different (Tukey's test: $P>0.05$). VF = two virgin females

caught during two nights. This experiment again showed the blend containing *Z*3-12:OAc plus *Z*5-12:OAc caught significantly more males than virgin females (Fig. 4). Moreover, trap catches of males to this blend were unaffected by the addition of the *E*3-12:OAc and/or *Z*5-14:OAc. Conversely, removal of *Z*5-12:OAc from the synthetic blends resulted in a significant decrease in attraction. In the final experiment, testing different blends of *Z*3-12:OAc and *Z*5-12:OAc, totals of 3,328 moths were caught. Increasing the amount of *Z*5-12:OAc from 5% to 100% relative to *Z*3-12:OAc showed a maximum catch at 5%, but 20% and greater amounts dramatically reduced trap catches (Fig. 5).

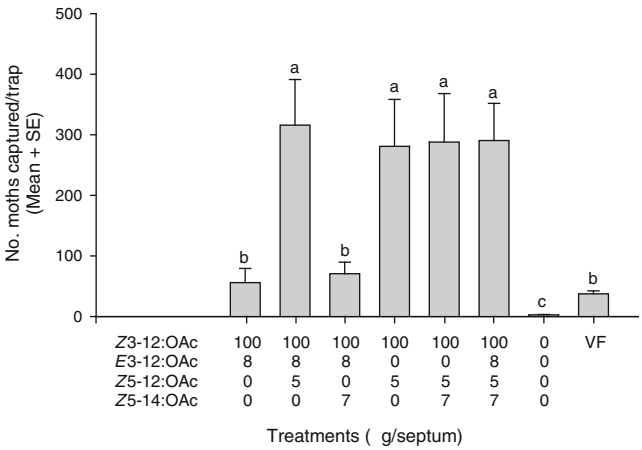


Fig. 4 Catches of male *Scrobipalpa salinella* in traps baited with *Z*3-12:OAc in binary, ternary, and quaternary combinations with *E*3-12:OAc, *Z*5-12:OAc, and *Z*5-14:OAc in glasswort fields in Gimje, Korea, 16–18 June 2010. Bars with the same letter are not significantly different (Tukey's test: $P>0.05$). VF = two virgin females

Discussion

This study demonstrates that *S. salinella* uses 100:5 blend of *Z*3-12:OAc and *Z*5-12:OAc as its sex pheromone. These two components have been identified in SPME collections and solvent extracts, but their relative ratios are markedly different from each other. Differences in pheromone composition between gland extracts and female volatiles have been reported from other moths, including *Sesia apiformis* (Francke et al., 2004), *Marmara gulosa* (McElfresh et al., 2009), and *Spodoptera exigua* (Acín et al., 2010), showing that this discrepancy is usual in the Lepidoptera. The considerable variability in relative ratio and small amount of *Z*3-12:OAc and *Z*5-12:OAc recovered from SPME samples may be attributable to the degradation and/or adsorption of acetate components by the moth bodies, the loose scales, and adsorption to the walls of the container during overnight collections, as observed in other moths (Baker et al., 1980; Millar and Rice, 1992). In field testing different blend ratios of *Z*3-12:OAc and *Z*5-12:OAc, the 100:5 blend, respectively, found in gland extracts caught ca. 35 times more *S. salinella* males than a 100:50 ratio similar to that found in SPME samples. This finding supports our previous observations that male moths are preferentially attracted to synthetic blends that mimic the ratios found in pheromone gland extracts of their conspecific females (Yang et al., 2008, 2011a, b).

*E*3-12:OAc was recovered in gland extracts of *S. salinella* females, but not in volatiles emitted by females. In field trials, the binary blend of *Z*3-12:OAc and *E*3-12:OAc was as attractive as virgin female moths, although it was significantly less attractive than the binary blend of *Z*3-12:OAc and *Z*5-12:OAc. This indicates that *E*3-12:OAc cross activated the peripheral pheromone receptor for *Z*5-12:OAc, the critical secondary pheromone com-

ponent (Priesner, 1986). There is some evidence for substitution of minor components by structural analogs of pheromone components (Inomata et al., 2005; Naka et al., 2007). In contrast, addition of *E*3-12:OAc at the level found in the glands of females did not affect captures of male moths to the primary binary blend of *Z*3-12:OAc and *Z*5-12:OAc, suggesting that *E*3-12:OAc is not part of a sex pheromone blend of this species. Similarly, *Z*5-14:OAc was detected in gland extracts, but its biological activity was not confirmed in field trials. Therefore, *E*3-12:OAc and *Z*5-14:OAc found in *S. salinella* pheromone glands may be present as by-products from the pheromone biosynthetic pathway that produces the major component, *Z*3-12:OAc.

Sex pheromones in the genus *Scrobipalpa* have been elucidated for only two species. The sex pheromone of *S. ocellatella* was reported to consist of *E*3-12:OAc as a single component (Renou et al., 1980). In another congeneric species, *S. heliopa,* females produce *E*3-13:OAc as the main pheromone component, which is highly attractive to conspecific males in the field (Baker et al., 1985). Thus, to date, *S. salinella* is the only *Scrobipalpa* species for which two components are essential for attraction of male moths.

*Z*3-12:OAc, the major component of the sex pheromone of *S. salinella*, has been discovered to be an attractant for two gelechiids, *Aproaerema anthyllidella* (Priesner, 1987) and *Aproaerema anthyllidella* (Reed and Chisholm, 1985), in field tests, but had not been identified from conspecific females. Conversely, Nesbitt et al. (1985) identified *Z*3-12:OAc in *Tecia* (formerly *Scrobipalpopsis*) *solanivora* as a minor component, and found an antagonistic effect of this compound on male attraction when it was mixed with the major component, *E*3-12:OAc. Our study with *S. salinella* is the first case in which *Z*3-12:OAc identified from females was attractive to males of the same species. Similarly, *Z*5-12:OAc, the critical component for attraction of male *S. salinella,* has been reported as an attractant in several gelechiid species, including *Caryocolum marmoreum*, *Chionodes fuscomaculella*, and *Gelechia sororculella* (El-Sayed, 2011). However, *Z*5-12:OAc has not been identified previously from pheromone glands of females in any gelechiid species. Thus, this is the first report of this compound as a sex pheromone component in the Gelechiidae.

In another field trial conducted during July and October of 2010 in glasswort fields at Gimje and Shinan, Korea, we captured only a few individuals of other insects in traps baited with a 100:5 blend of *Z*3-12:OAc and *Z*5-12:OAc, indicating that this mixture is a sensitive and selective attractant for monitoring *S. salinella*. Studies are underway to evaluate the effect of pheromone-based mating disruption against this species under field conditions.

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useful mating disruptant for integrated pest management (IPM) of the moth in pear orchards. In Gracillariidae, sex pheromones have been identified from two *Caloptilia*, one *Conopomorpha*, and one *Marmara* species (Gracillariinae); one *Cameraria* and 11 *Phyllonoryctor* species (Lithocolletinae) and one *Phyllocnistis* species (Phyllocnistinae) (Ando, 2011; El-Sayed, 2011).

The components are mono-, di-, and trienyl compounds with a functional group at the terminus of a C_{12}–C_{16} straight chain. The positions of the double bonds vary, and several compounds with a characteristic polyenyl system have been reported as follows: a C_{14} 4,10-diene in *Phyllonorycter ringoniella* (Sugie et al., 1986; Ujiye et al., 1986), C_{14} 8,10-dienes in *Cameraria ohridella* (Svatoš et al., 1999) and *Phyllonorycter emberizaepenella* (Mozûraitis et al., 2002), a C_{14} 10,12-diene in *Phyllonorycter crataegella* (Ferrao et al., 1998), C_{16} 4,6,10-trienes in *Conopomorpha cramerella* (Beevor et al., 1986), and a C_{16} 7,11-diene and a C_{16} 7,11,13-triene in *Phyllocnistis citrella* (Mafi et al., 2005; Leal et al., 2006; Moreira et al., 2006; Lapointe et al., 2006). Pheromones secreted by the *Spulerina* species, however, have never been investigated. We became interested in the pheromone structure of *S. astaurota*, and then found 9,11-dienes from the female moths. Results of field evaluation of synthetic pheromone components appear likely to lead to a reliable monitoring method.

Methods and Materials

Insects and Pheromone Extract Larvae of *S. astaurota* overwinter and pupate in May. Pupae were collected from Asian-pear orchards in Tottori Prefecture (Japan) from late May to early June 2009, and were reared in plastic boxes in the laboratory (25±1°C, 16L-8D). After emergence, male and female moths were sexed and individually placed into 5-ml vials. Abdominal tips of fifty 2- to 4-d-old female moths were cut and extracted with hexane for 1 hr, filtrated, and kept in a freezer at −30°C until use. A portion of the female extract was used for bioassays and chemical analysis without purification. Column chromatography of an aliquot of the extract (30 female equivalents, FE) was conducted on Florisil (500 mg) using 5, 10, 20, and 30% ether in hexane (3 ml each) as an eluant.

Derivatization of Pheromone Components A 25-FE aliquot of crude extract was dissolved in CH_2Cl_2 (10 μl), and treated with a CH_2Cl_2 solution of 4-methyl-1,2,4-triazoline-3,5-dione (MTAD, 10 mg/ml, 40 μl) for 30 min at room temperature (Young et al., 1990). After changing the solvent to hexane, the MTAD adducts were analyzed by gas chromatography coupled with mass spectrometry (GC-MS). To confirm the structure determination of the natural pheromone components, a mixture of authentic 9,11-tetradecadienyl compounds (250 ng each) was treated with the MTAD solution in the same manner as for the natural pheromone, and the products were analyzed by GC-MS.

Analytical Instruments The extracts of females and synthetic compounds were analyzed by GC with an electroantennographic (EAG) detector (GC-EAD). The GC was equipped with a DB-23 capillary column (0.25 mm×30 m, 0.25 μm, J & W Scientific, Folsom, CA, USA), and the effluent from the column was split into two lines, which led to a flame ionization detector (FID) and EAD at a ratio of 1:1 (Inomata et al., 2005). The oven temperature was maintained at 80°C for 1 min, programmed at 8°C/min to 210°C, and held for 10 min. Electron ionization (EI, 70 eV) GC-MS was conducted with an HP5975 mass spectrometer system (quadrupole type, Hewlett Packard) equipped with a split/splitless injector, and the same DB-23 column as the GC-EAD analysis or an HP-5 column (0.25 mm×30 m, 0.25 μm, Hewlett-Packard, Wilmington, DE, USA). The flow rate of the carrier gas (He) was 1.0 ml/min for all GC analyses, and the temperature of GC-inlet was 220°C. The oven temperature for all GC-MS analyses was the same as that for GC-EAD except for the MTAD derivatization experiment, for which the oven temperature of adducts was maintained at 100°C for 2 min, programmed at 15°C/min to 280°C, and held for 15 min. ^{1}H and ^{13}C NMR spectra were recorded by a Jeol Delta 2 Fourier transform spectrometer (JEOL Ltd., Tokyo, Japan) at 399.8 and 100.5 MHz, respectively, in $CDCl_3$ solutions containing TMS as an internal standard.

Synthesis of 9,11-Tetradecadienyl Compounds Four geometrical isomers of C_{14} 9,11-dienes were synthesized starting from 1,8-octanediol (**1**) or 1,9-nonanediol (**5**) as shown in Fig. 1. Experimental details are shown in supplements.

(9*Z*,11*Z*)-Isomers (Scheme A): After one hydroxyl group of the commercially available diol **1** was protected with 3,4-dihydro-2*H*-pyran, another hydroxyl group was iodized by a treatment with a mixture of I_2, triphenylphosphine (PPh_3), and imidazole to yield a THP ether of 8-iodooctan-1-ol (**2**). A coupling reaction of **2** with lithium trimethylsilylacetylide in a mixed solvent of THF and HMPA and desilylation of the product with potassium carbonate in methanol produced a THP ether of 9-decyn-1-ol (**3**). Sonogashira-Hagihara coupling (Sonogashira et al., 1975) between **3** and (*Z*)-1-bromo-1-butene, which was prepared from (*E*)-2-pentenoic acid (Mori and Brevet, 1991; Mori, 2009), furnished a THP ether of (*Z*)-11-tetradecen-9-yn-1-ol (**4**). ^{1}H NMR analysis of **4** confirmed the *Z* configuration of the double bond at the 11-position; i.e., olefinic proton

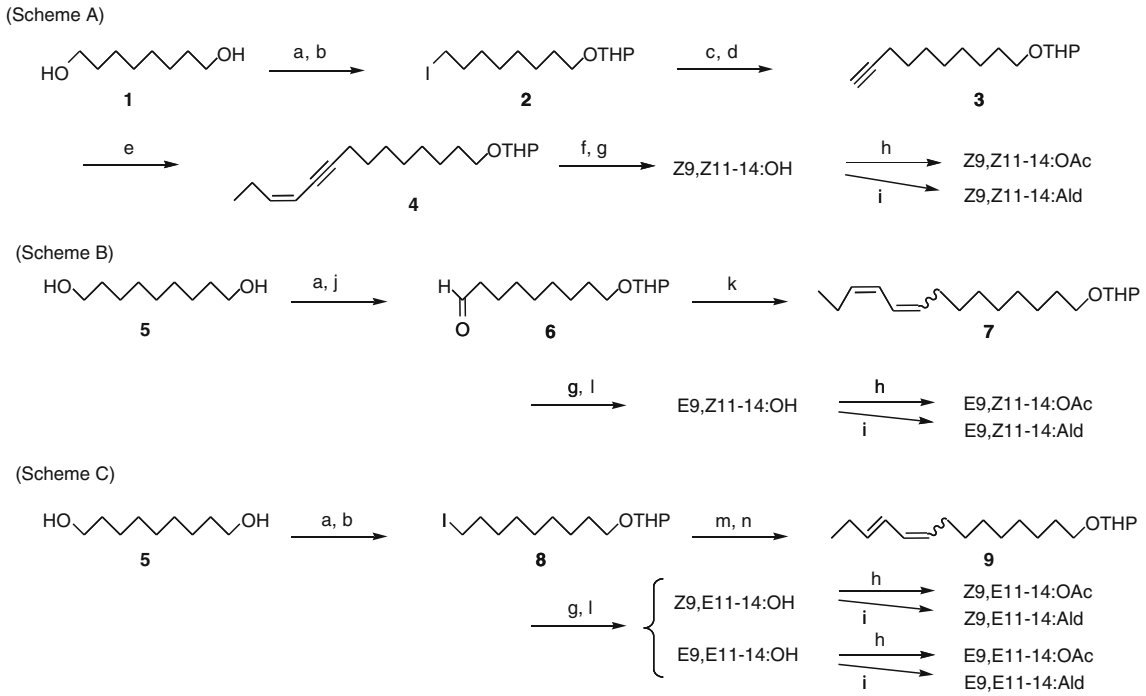


Fig. 1 Synthetic schemes for 9,11-tetradecadienyl compounds (alcohols, acetates, and aldehydes); (9*Z*,11*Z*)-isomer (Scheme A), (9*E*,11*Z*)-isomer (Scheme B), and (9*Z*,11*E*)- and (9*E*,11*E*)-isomers (Scheme C). Reagents: a, 3,4-dihydro-2*H*-pyran/*p*-TsOH/CH_2Cl_2; b, I_2-PPh_3/imidazole/THF; c, TMSC≡CH/BuLi/THF-HMPA; d, K_2CO_3/MeOH; e, (*Z*)-1-bromobutene/$Pd(PPh_3)_4$/CuI/$PrNH_2$/benzene; f, (1) $BH(C_6H_{11})_2$/THF, (2) AcOH, (3) $NaOH/H_2O_2$ (30%); g, *p*-TsOH/EtOH; h, Ac_2O/pyridine; i, PCC/CH_2Cl_2; j, $(COCl)_2$/DMSO/Et_3N/CH_2Cl_2; k, (*Z*)-2-pentenylenetriphenylphosphorane/THF; l, column chromatography with SiO_2-$AgNO_3$; m, Ph_3P/Δ; n, (1) BuLi/THF, (2) (*E*)-2-pentenal

resonating at δ 5.40 and 5.80 ppm showed a similar coupling constant (J=10.5 Hz) as that usually detected for (*Z*)-isomers of disubstituted alkenes. The triple bond of **4** was selectively reduced to a (*Z*)-double bond by hydroboration-protonolysis using dicyclohexylborane, and deprotection of the produced THP ether yielded (9*Z*,11*Z*)-9,11-tetradecadien-1-ol (Z9,Z11-14:OH). ^{1}H NMR δ ppm: 0.99 (3H, t, J=7.5 Hz, CH_3CH$_2$), ~1.3 (10H, m), 1.55 (2H, m, CH_2CH$_2$OH), 2.17 (4H, m, CH_2CH=CHCH=CHCH_2), 3.61 (2H, t, J=6.5 Hz, CH_2OH), 5.44 (2H, m, C*H*=CHCH=C*H*), 6.23 (2H, m, CH=C*H*C*H*=CH), ^{13}C NMR δ ppm: 14.2, 20.8, 25.8, 27.5, 29.2, 29.4, 29.5, 29.6, 32.8, 62.9, 123.0, 123.5, 132.1, 133.6. The acetate (Z9,Z11-14:OAc) and aldehyde derivative (Z9,Z11-14:Ald) were synthesized from Z9,Z11-14:OH by treatment with acetic anhydride in pyridine and with pyridinium chlorochromate (PCC) in CH_2Cl_2, respectively.

(9*E*,11*Z*)-Isomers (Scheme B): Half protection of diol (**5**) with 3,4-dihydro-2*H*-pyran and Swern oxidation of the remaining hydroxyl group produced a THP ether of 9-hydroxynonanal (**6**). A bromide derived from (*Z*)-2-penten-1-ol was converted to (*Z*)-2-pentenyltriphenylphosphorium bromide by heating with PPh_3. The aldehyde **6** was coupled with the ylide, which was prepared from the phosphonium salt by the treatment with butyllithium (BuLi), to yield a THP ether of 9,11-tetradecadien-1-ol (**7**). NMR analysis revealed that **7** was a mixture of (9*E*,11*Z*)- and (9*Z*,11*Z*)-isomers in a ratio of about 1:1. After deprotection, Z9,Z11-14:OH and the (9*E*,11*Z*)-isomer (E9,Z11-14:OH) were separated by column chromatography utilizing silica gel impregnated with $AgNO_3$. NMR data of E9,Z11-14:OH; ^{1}H NMR δ ppm: 0.99 (3H, t, J=7.5 Hz, CH_3CH$_2$), ~1.3 (10H, m), 1.56 (2H, m, CH_2CH$_2$OH), 2.08 (2H, m, CH_2CH=CH), 2.18 (2H, m, CH_2CH=CH), 3.63 (2H, t, J=6.5 Hz, CH_2OH), 5.30 (1H, dt, J=11, 7.5 Hz, C*H*=CHCH=CH), 5.65 (1H, dt, J=15, 7 Hz, CH=CHCH=C*H*), 5.91 (1H, dd, J=11, 11 Hz, CH=C*H*CH=CH), 6.30 (1H, dd, J=15, 11 Hz, CH=CHC*H*=CH); ^{13}C NMR δ ppm: 14.3, 21.0, 25.7, 29.2, 29.4 (×2), 29.5, 32.8, 32.9, 63.1, 125.5, 128.0, 131.7, 134.7. The acetate (E9,Z11-14:OAc) and aldehyde derivative (E9,Z11-14:Ald) were synthesized from E9,Z11-14:OH.

(9*Z*,11*E*)- and (9*E*,11*E*)-Isomers (Scheme C): In a similar synthetic procedure from C_8 diol **1** to **2**, C_9 diol **5** was converted to a THP ether of 9-iodononan-1-ol (**8**). The iodide **8** was heated with PPh_3, and a produced phosphonium salt was treated with BuLi to make an ylide, which was coupled with (*E*)-2-pentenal to yield a THP ether of 9,11-tetradecadien-1-ol (**9**). NMR analysis revealed that **9** was a mixture of (9*Z*,11*E*)- and (9*E*,11*E*)-isomers in a ratio of about 7:3. After deprotection, two alcohols, Z9,E11-14:OH and E9,E11-14:OH, were separated by column chro-

matography utilizing silica gel impregnated with $AgNO_3$. NMR data of Z9,E11-14:OH; ^{1}H NMR δ ppm: 1.01 (3H, t, J=7.5 Hz, CH_3CH$_2$), ~1.3 (10H, m), 1.54 (2H, m, CH_2CH$_2$OH), 2.12 (4H, m, CH_2CH=CHCH=CHCH_2), 3.58 (2H, t, J=6.5 Hz, CH_2OH), 5.28 (1H, dt, J=11, 7.5 Hz, CH=CHCH=CH), 5.68 (1H, dt, J=15, 7 Hz, CH=CHCH=CH), 5.93 (1H, dd, J=11, 11 Hz, CH=CHCH=CH), 6.29 (1H, dd, J=15, 11 Hz, CH=CHCH=CH); ^{13}C NMR δ ppm; 13.7, 25.8, 25.9, 27.7, 29.3, 29.5, 29.6, 29.8, 32.7, 62.7, 124.8, 128.7, 130.0, 136.1. NMR data of E9, E11-14:OH; ^{1}H NMR δ ppm: 0.99 (3H, t, J=7.5 Hz, CH_3CH$_2$), ~1.3 (10H, m), 1.56 (2H, m, CH_2CH$_2$OH), 2.06 (4H, m, CH_2CH=CHCH=CHCH_2), 3.62 (2H, t, J=6.5 Hz, CH_2OH), 5.59 (2H, m, CH=CHCH=CH), 6.00 (2H, m, CH=CHCH=CH); ^{13}C NMR δ ppm: 13.7, 25.6, 25.7, 29.1, 29.39, 29.43, 29.5, 32.6, 32.8, 63.0, 129.4, 130.4, 132.4, 133.9. The acetates (Z9,E11-14:OAc and E9,E11-14:OAc) and aldehyde derivatives (Z9,E11-14:Ald and E9,E11-14:Ald) were synthesized from the corresponding alcohols.

Field Trapping of Male Moths The synthetic pheromone constituents dissolved in hexane (100 µl) were incorporated into rubber septa (white rubber, O.D. 8 mm; Sigma-Aldrich, USA) used as dispensers. Each lure was placed at the center of a sticky board trap (SE-trap®, 30×27 cm bottom plate with a roof; Sankei Chemical Co., Ltd., Kagoshima, Japan), and hung separately in Asian-pear orchards at intervals of 10 and 1.5 m height. Four experiments (A–D) were conducted in Tottori Prefecture in 2010 and 2011, and the number of captured males was counted weekly. In each field test, the replicated data were log-transformed before analysis by Tukey-Kramer's HSD test (Sokal and Rohlf, 1995). The level of significance in all tests was set at 5%.

Results

Structure Elucidation of Pheromone Components GC–EAD analyses of a crude pheromone extract of *S. astaurota* females showed three EAG-active components (Fig. 2). Of the three, the male antenna responded most to **III**. Compounds **I** and **II** were detected in the 10% ether fraction of the crude extract on a Florisil column, indicating an aldehyde and acetate, and **III** eluted in the 30% ether fraction as would an alcohol. Next, the crude extract was analyzed by GC-MS. The total ion chromatogram (TIC) and mass spectra of **II** and **III** (Fig. 3), exhibited M^+ ions at *m/z* 252 and 210 consistent with tetradecadienyl acetate and the parent alcohol, respectively. The chromatographic behavior with relatively long retention times on a polar GC column suggested conjugated dienyl structures, and characteristic fragment ions at *m/z* 82 and 95 in both spectra indicated two double bonds located at the same 9- and 11-positions (Ando et al., 1988; Ando and Yamakawa, 2011). Since **I** and an unknown substance were eluted together on the GC-MS analysis, no clear mass spectrum of **I** was recorded. However, mass chromatograms monitoring some characteristic ions for 9,11-tetradecadienal confirmed the occurrence of this compound in the crude pheromone extract. The chromatogram at *m/z* 67 suggested that females produce **I**–**III** in a ratio of about 1:2:20, respectively. The double bond positions were confirmed by GC-MS analysis

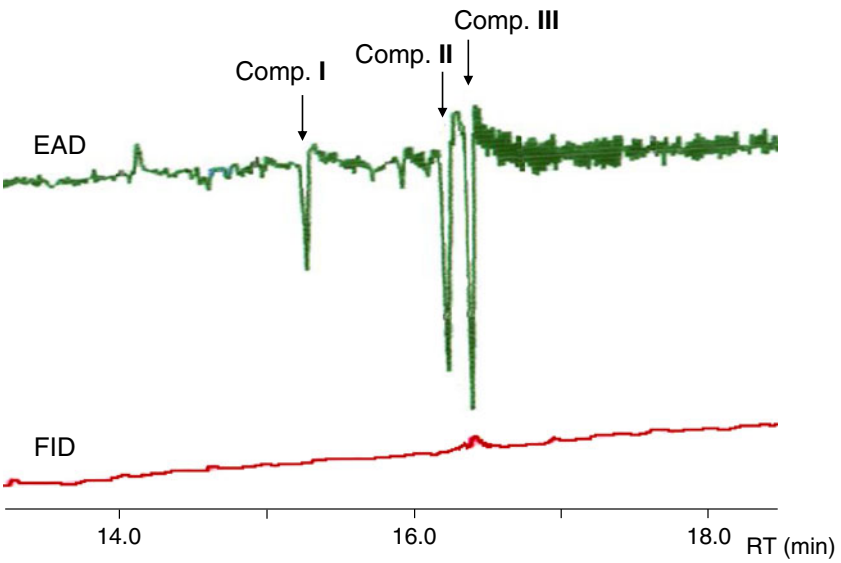


Fig. 2 GC-EAD analysis of the sex pheromone components of *Spulerina astaurota* females. Analysis of a crude pheromone extract from the female (0.5 FE), indicating three EAG-active components (**I**, RT 15.2 min; **II**, RT 16.2 min; and **III**, RT 16.4 min)

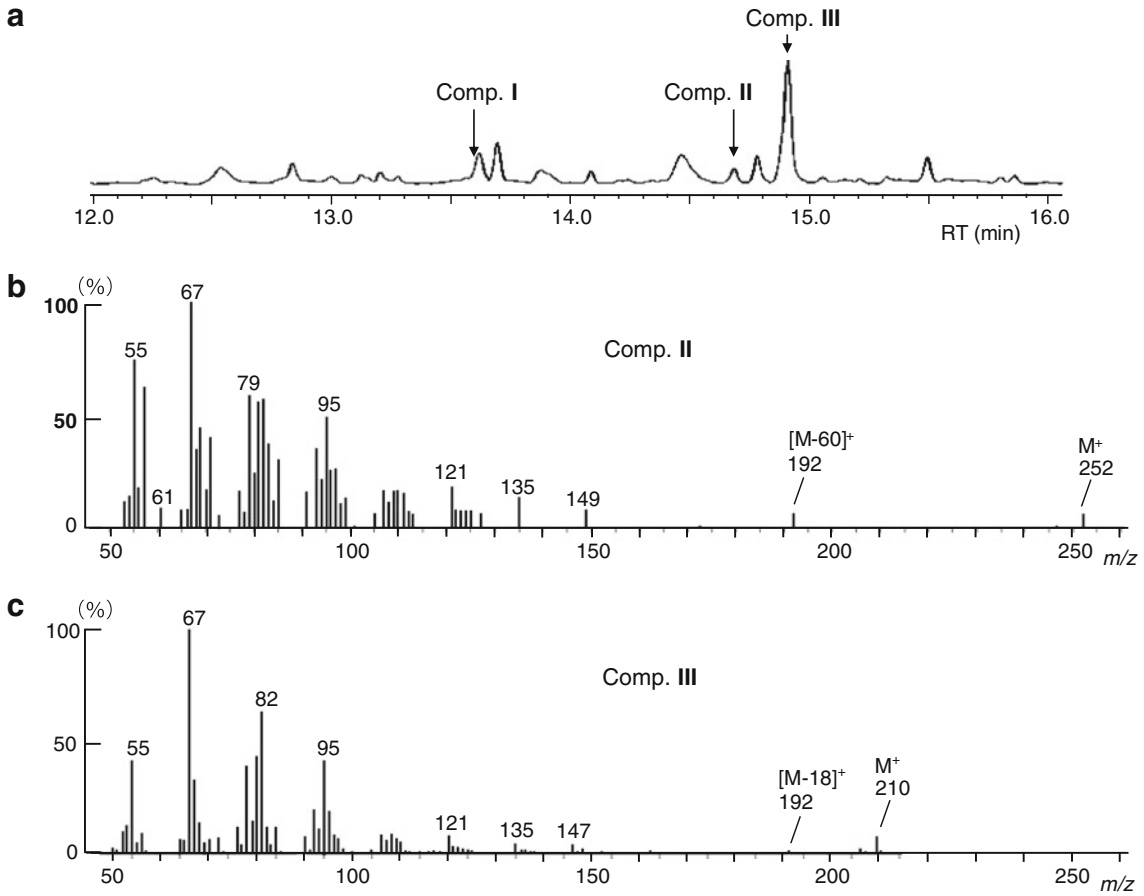


Fig. 3 GC-MS analysis of a crude pheromone extract from *Spulerina astaurota* females (3.5 FE). **a**, Total ion chromatogram (TIC); **b**, mass spectrum of **II** (Z9,Z11-14:OAc, RT 14.68 min); and **c**, mass spectrum of **III** (Z9,Z11-14:OH, RT 14.91 min). Mass chromatograms of M^+ at *m*/*z* 208 and some characteristic fragment ions of tetradecadienal (ions at *m*/*z* 67, 82, and 95) indicated an occurrence of **I** (Z9,Z11-14:Ald, RT 13.63 min) in the extract

of the crude pheromone extract (25 FE) after a treatment with MTAD. This analysis showed the formation of an MTAD adduct (RT 15.48 min) derived from **III**, the major pheromone component in the gland extract. The mass spectrum exhibited an M^+ ion at *m*/*z* 323, and fragment ions at *m*/*z* 294 and 194, characteristic for the adduct of 9,11-tetradecadien-1-ol.

Identification with Authentic Compounds All geometrical isomers of 9,11-tetradecadienyl compounds were synthesized by three different schemes, as shown in Fig. 1. (9*Z*,11*Z*)-Isomers were stereoselectively synthesized via a (*Z*)-9-en-11-ynyl intermediate (**4**), which was partially reduced to the conjugated diene by hydroboration (Scheme A). A mixture of (9*E*,11*Z*)- and (9*Z*,11*Z*)-isomers was prepared utilizing an ylide derived from (*Z*)-1-bromo-2-pentene (Scheme B). Another mixture of (9*Z*,11*E*)- and (9*E*,11*E*)-isomers was synthesized via Wittig coupling with (*E*)-2-pentenal as a key reaction (Scheme C). The two isomers in each mixture were separable, and their chemical structures were confirmed by NMR analyses. The (9*E*,11*Z*)- and (9*Z*,11*E*)-isomers showed four olefinic protons with a different chemical shift. Configuration of the double bonds was revealed by a coupling constant; i.e., 11 Hz for the *Z* configuration and 15 Hz for the *E* configuration. Although the coupling constant was not clear in the (9*Z*,11*Z*)- and (9*E*,11*E*)-isomers, allylic carbons resonated with crucially different chemical shifts reflecting the configuration; i.e., 20.8 and 27.5 ppm for Z9,Z11-14:OH and 25.6 and 32.6 ppm for E9,E11-14:OH.

Table 1 shows the RT of each isomer of 9,11-tetradecadienyl compounds (aldehyde, acetate, and alcohol) analyzed by GC-MS on two different columns, a strongly polar column (DB-23) and a weakly polar column (HP-5). While the RT of the aldehyde was the shortest among the three compounds in either column, the elution order of the acetate and alcohol changed according to the polarity of the column. Four geometrical isomers of each compound separately eluted from both columns in the same order of (9*Z*,11*E*)-, (9*E*,11*Z*)-, (9*Z*,11*Z*)-, and (9*E*,11*E*)-isomers. RTs

of three natural pheromone components coincided well with those of the (9*Z*,11*Z*)-isomers, indicating **I**, **II,** and **III** to be Z9,Z11-14:Ald, Z9,Z11-14:OAc, and Z9,Z11-14:OH, respectively. The GC-MS analysis of the crude pheromone extract indicated that one female possessed about 2 ng of Z9,Z11-14:OH.

Field Evaluation of the Synthetic Pheromone Since the *S. astaurota* females produce Z9,Z11-14:OH (**III**) as the major pheromone component, the activities of synthetic pheromone lures including Z9,Z11-14:OH alone and mixed with Z9,Z11-14:OAc (**II**) in different ratios (totally 0.5 mg for each septum) were examined first (Experiment A). The results from the 2010 tests are shown in Table 2. While Z9, Z11-14:OH without Z9,Z11-14:OAc attracted some males, the activities of the 7:3 and 5:5 mixtures were significantly greater than the single component lure. No males were attracted by the lure baited solely with Z9,Z11-14:OAc, indicating that the acetate occurring as a minor pheromone component is an effective synergist. Next, activities of four geometrical isomers were compared in order to confirm the configuration of the natural pheromone components (Experiment B). The 7:3 mixture of Z9,Z11-14:OH and Z9, Z11-14:OAc more effectively attracted male moths than did 7:3 mixtures of the dienyl alcohol and acetate with other configurations, as shown in Table 3.

Based on results of the field tests in 2010, the effect of Z9,Z11-14:Ald (**I**) on the activity of the 7:3 mixture of Z9, Z11-14:OH (0.35 mg/septum) and Z9,Z11-14:OAc (0.15 mg/septum) was examined in 2011 (Experiment C). Male attraction by the three-component lures including the aldehyde (0.05–0.5 mg/septum) was not significantly different from that by the two-component lures (Table 4). The number of captured males/trap/night decreased extraordinary in 2011, indicating a lower population density than that in 2010.

Although no geometrical isomers of Z9,Z11-14:OH were found in the gland extract, their contamination in the synthetic pheromone might easily have happened. To apply the synthetic lure as a monitoring tool, it is important to understand their effects on male attraction. Each isomer of Z9,Z11-14:OH was added to the 7:3 mixture of Z9,Z11-14: OH and Z9,Z11-14:OAc, and the lures were evaluated in the field (Experiment D). Significant differences were not observed between the lure unmixed and mixed with one geometrical isomer as a third component even at a ratio of 7:3:4 (Table 5).

Discussion

GC-EAD and GC-MS analyses of the pheromone gland extract of *Spulerina astaurota* females revealed three EAG-active components; 9,11-tetradecadien-1-ol, its acetate, and an aldehyde derivative (Figs. 2 and 3). Comparing their chromatographic behaviors to those of the synthetic standards of all geometrical isomers confirmed the 9*Z*,11*Z* configuration of the natural components (Table 1). Therefore, we concluded Z9,Z11-14:OH, Z9,Z11-14:OAc, and Z9,Z11-14:Ald as candidates of the *S. astaurota* pheromone components. Their biological activities were evaluated in a series of field-trap experiments using synthetic compounds (Tables 2, 3 and 4). The male moths were attracted to a lure baited with Z9,Z11-14:OH alone, and Z9,Z11-14:OAc strongly synergized attraction. Among the lures tested, the

Table 1 Chromatographic behaviour of synthetic 9,11-tetradecadienyl compounds and natural pheromone components of *Spulerina astaurota* females on GC-capillary columns [a]

Compound	Retention time (RT, min)					
	DB-23 column [b]			HP-5 column [c]		
	Aldehyde	Acetate	Alcohol	Aldehyde	Acetate	Alcohol
(9*Z*,11*E*)-Isomer	13.48	14.58	14.79	13.97	16.60	14.86
(9*E*,11*Z*)-Isomer	13.60	14.65	14.88	14.09	16.71	14.98
(9*Z*,11*Z*)-Isomer	13.63	14.68	14.91	14.20	16.80	15.12
(9*E*,11*E*)-Isomer	13.66	14.74	14.94	14.24	16.87	15.16
Natural component [d]	13.63	14.68	14.91	14.19	16.80	15.12

[a] Each compound was analyzed by GC-MS equipped with a DB-23 column (0.25 mm ID×30 m) and an HP-5 column (0.25 mm ID×30 m). The column temperature program was 80°C for 2 min, 8°C/min to 210°C, and 210°C for 10 min

[b] Kováts retention index values of (9*Z*,11*Z*)-isomers: Z9,Z11-14:Ald, 2161; Z9,Z11-14:OAc, 2278; Z9,Z11-14:OH, 2297

[c] Kováts retention index values of (9*Z*,11*Z*)-isomers: Z9,Z11-14:Ald, 1669; Z9,Z11-14:OAc, 1861; Z9,Z11-14:OH, 1734

[d] Aldehyde (Comp. **I**, Z9,Z11-14:Ald), acetate (Comp. **II**, Z9,Z11-14:OAc), and alcohol (Comp. **III**, Z9,Z11-14:OH). RT of the Comp. **I** was determined by mass chromatogram monitoring the ion at *m*/*z* 67

Table 2 Attraction of *Spulerina astaurota* males by lures baited with synthetic Z9,Z11-14:OH and Z9,Z11-14:OAc mixed in a different ratio (Experiment A) [a]

Lure (mg/rubber septum) [b]			Captured males	
Z9,Z11-14:OH	Z9,Z11-14:OAc	Ratio	/trap/night [c]	Total
0.50	0	10:0	2.43±1.05 b	153
0.45	0.05	9:1	2.51±0.80 b	158
0.35	0.15	7:3	5.97±2.79 a	376
0.25	0.25	5:5	5.40±0.94 a	340
0.05	0.45	1:9	0.75±0.26 c	47
0	0.50	0:10	0.0	0
0	0		0.02±0.02 d	1

[a] Tested using three traps for each lure from June 22 to July 13, 2010 in an Asian-pear orchard of Tottori city and Hokuei town in Tottori Prefecture

[b] Z9,Z11-14:OH (Comp. **III**), Z9,Z11-14:OAc (Comp. **II**)

[c] Mean±SE. Values within each test followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer test

mixture of Z9,Z11-14:OH and Z9,Z11-14:OAc in a ratio of 7:3 exhibited the strongest attraction. On the other hand, the addition of Z9,Z11-14:Ald to the mixture did not significantly increase the number of males attracted. The role of Z9,Z11-14:Ald in this communication system is unclear.

Some other dienyl pheromones are known for the species in Gracillariidae, but the 9,11-dienes have not been identified from species in this family (Ando, 2011; El-Sayed, 2011). More than 200 Gracillariidae species occur in Japan, including several species in the *Spulerina* that mine into specific host plants, such as *S. corticicola* that attacks pine trees. 9,11-Tetradecadienyl compounds, however, are not novel pheromone components of lepidopteran insects. Interestingly, females of the currant shoot borer, *Lampronia capitella* (Incurvarioiidae), also produced the same three components, Z9,Z11-14:OH, Z9,Z11-14:OAc, and Z9,Z11-14:Ald, and the mixture in a ratio of 100:26:13 attracted the males (Löfstedt et al., 2004). The two micro-lepidopteran species are classified in different groups even at a superfamily; i.e., *S. astaurota* in Gracillarioidea and *L. capitella* in Incurvarioidea. In addition to these two species, Z9,Z11-14:OAc has been identified from *Idaea aversata* and *I. straminata* (Geometridae) as a main pheromone component (Zhu et al., 1996).

Table 3 Attraction of *Spulerina astaurota* males by lures baited with 9,11-tetradecadienyl alcohol and acetate mixed in a 7:3 ratio (Experiment B) [a]

Lure (mg/rubber septum) [b]		Captured males	
Alcohol (0.35 mg)	Acetate (0.15 mg)	/trap/night [c]	Total
Z9,Z11-14:OH	Z9,Z11-14:OAc	63.05±24.81 a	2,648
Z9,E11-14:OH	Z9,E11-14:OAc	3.76±2.41 b	158
E9,Z11-14:OH	E9,Z11-14:OAc	1.90±0.89 b	80
E9,E11-14:OH	E9,E11-14:OAc	1.98±1.15 b	83
none	none	0.10±0.10 c	4

[a] Tested using three traps for each lure from September 1 to September 15, 2010 in an Asian-pear orchards of Hokuei town in Tottori Prefecture

[b] Z9,Z11-14:OH (Comp. **III**), Z9,Z11-14:OAc (Comp. **II**)

[c] Mean±SE. Values within each test followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer test

Table 4 Attraction of *Spulerina astaurota* males by lures baited with synthetic Z9,Z11-14:OH, Z9,Z11-14:OAc, and Z9,Z11-14:Ald mixed in a different ratio (Experiment C) [a]

Lure (mg/rubber septum) [b]				Captured males	
Z9,Z11-14:OH	Z9,Z11-14:OAc	Z9,Z11-14:Ald	Ratio	/trap/night [c]	Total
0.35	0.15	0	7:3:0	0.80±0.19 a	28
0.35	0.15	0.05	7:3:1	1.11±0.44 a	39
0.35	0.15	0.20	7:3:4	1.49±0.65 a	52
0.35	0.15	0.50	7:3:10	1.11±0.37 a	39
0	0	0.50	0:0:10	0.03±0.03 b	1
0	0	0		0±0	0

[a] Tested using five traps for each lure from July 5 to July 12, 2011 in an Asian-pear orchard of Tottori city in Tottori Prefecture

[b] Z9,Z11-14:OH (Comp. **III**), Z9,Z11-14:OAc (Comp. **II**), Z9,Z11-14:Ald (Comp. **I**)

[c] Mean±SE. Values within each test followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer test

Table 5 Attraction of *Spulerina astaurota* males by the synthetic pheromone (Z9,Z11-14:OH and Z9,Z11-14:OAc) mixed with a geometrical isomer of the alcohol component (Experiment D) [a]

Lure (mg/rubber septum) [b]		Captured males	
Z9,Z11-14:OH (0.35)+Z9,Z11-14:OAc (0.15)	Ratio	/trap/night [c]	Total
+ none	7:3:0	0.63±0.52 a	53
+ Z9,E11-14:OH (0.05)	7:3:1	0.52±0.38 a	44
+ Z9,E11-14:OH (0.2)	7:3:4	0.80±0.64 a	67
+ E9,Z11-14:OH (0.05)	7:3:1	0.58±0.41 a	49
+ E9,Z11-14:OH (0.2)	7:3:4	0.90±0.72 a	76
+ E9,E11-14:OH (0.05)	7:3:1	1.05±0.89 a	88
+ E9,E11-14:OH (0.2)	7:3:4	0.55±0.31 a	46
control		0±0	0

[a] Tested using four traps for each lure from June 14 to July 5, 2011 in an Asian pear orchard of Hokuei town in Tottori Prefecture

[b] Z9,Z11-14:OH (Comp. **III**), Z9,Z11-14:OAc (Comp. **II**)

[c] Mean±SE. Values within each test followed by a different letter are significantly different at $P<0.05$ by Tukey-Kramer test

Several lepidopteran sex pheromones including geometrical isomers of (9*Z*,11*Z*)-9,11-tetradecadienyl compounds also have been reported (Ando, 2011; El-Sayed, 2011). Z9, E11-14:OAc is a well-known pheromone component of Noctuidae species (*Spodoptera eridania*, *S. littoralis*, and *S. litura*), and this compound has been identified from a Pyralidae species, *Dioryctria abietella*. The aldehyde derivative with 9*Z*,11*E* configuration was produced by another Pyralidae species (*Ectomyelois ceratoniae*), and an Elachistidae species (*Stenoma cecropia*). Furthermore, E9, E11-14:OAc is secreted by a Tortricidae species, *Epiphyas postvittana*. Thus, taxonomically unrelated species have evolved to produce the same conjugated dienyl compounds in the Lepidoptera.

Biosynthesis of the pheromone components have been studied in *S. littoralis* and *E. postvittana*. In the former species, Z9,E11-14:OAc was biosynthesized by Δ9-desaturation of (*E*)-11-tetradecenyl intermediate (Dunkelblum and Kehat 1987; Martinez et al., 1990). In contrast, E9,E11-14:OAc was biosynthesized by Δ11-desaturation of (*E*)-9-tetradecenyl intermediate, which was produced by β-oxidation of (*E*)-11-hexadecenoate, in the latter species (Foster and Roelofs, 1990). Two different pathways were proposed for 9,11-dienes with a different configuration. To date, no biosynthetic studies have focused on lepidopteran pheromones with *Z*,*Z* configured conjugated dienyl compounds; therefore, (9*Z*,11*Z*)-9,11-dienes of the *S. astaurota* females might be interesting in this regard.

The *S. astaurota* adults appear twice a year; from June through July and in September. In our 2010 field tests, we caught approximately 4,000 male moths, indicating that the synthetic pheromone is powerfully attractive and potentially useful for accurately detecting mating activity of this pest. In field experiment D, the activity of the synthetic pheromone was not decreased by admixture of geometrical isomers of Z9,Z11-14:OH (Table 5). This result indicates that high purity of the synthetic dienyl compound is not necessary to make a lure for monitoring. Regrettably, some data in Tables 3, 4 and 5 vary with a large standard error. Each of Experiments B through D was carried out in one village, but the traps baited with the same lure were placed in different orchards located at some distance apart. It is possible that the population densities in the orchards used for the field tests were different, resulting in the variable attraction. These results also suggest that the flight area for each male moth was not extensive. The number of captured males in 2011 was extraordinary small, as shown in Tables 4 and 5, indicating the possibility of a lower population density in 2011 than in 2010. Future work utilizing the synthetic pheromone of *S. astaurota* should clarify the ecology of this species, including the extent to which the number of trapped males correlates with larval damage.

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high sugar resource, with cascading impacts on the ecosystem (Blüthgen and Fiedler, 2004; Blüthgen et al., 2004). Honeydew also has facilitated the establishment and spread of some invasive species such as yellow crazy ants (*Anoplolepis gracilipes*) on Christmas Island (O'Dowd et al., 2003) and *Vespula* wasps in New Zealand (Harris et al., 1991). Other nutrient-rich food resources, such as extra floral nectaries (EFNs) and nectar, influence ant preferences via sugar and amino acid composition (González-Teuber and Heil, 2009a, b). The first step towards understanding the ecological processes influenced by honeydew is to describe its chemical composition.

In New Zealand, there are two endemic genera of coelostomidiid scale insects that produce droplets of honeydew at the end of a long waxy anal filament (Morales, 1991). These scale insects form the basis for at least three honeydew-influenced native forest communities: *Ultracoelostoma* spp.–southern beech (*Nothofagus*) forest; *Coelostomidia zealandica*–coastal forest; *C. wairoensis*–kānuka (*Kunzea*) scrub (Table 1). The dominant above-ground animal honeydew consumers differ among these forest types, with birds and insects utilizing *Ultracoelostoma* spp. honeydew in southern beech forests (Gaze and Clout, 1983; Beggs and Wardle, 2006), geckos and insects utilizing

Table 1 Summary of three honeydew-influenced forest communities in New Zealand

			
	Beech forest	Kānuka scrub	Coastal forest
Main host species:	Beech (*Nothofagus* spp.)	Kānuka (*Kunzea ericoides*)	Ngaio (*Myoporum laetum*) & Karo (*Pittosporum crassifolium*)
Main scale insect species:	*Ultracoelostoma* spp.	*Coelostomidia wairoensis*	*Coelostomidia zealandica*
Scale insect distribution:	Widespread	Widespread	Widespread
Scale insect infestation pattern:	Heavy infestation common	Heavy infestation common	Heavy infestation rare
Main native animal honeydew consumers:	Birds & insects	Moths & other insects	Reptiles & insects
Threats:	*Vespula* invasion	*Vespula* invasion	Habitat fragmentation
Main references:	(Beggs and Wardle 2006)	(Gardner -Gee and Beggs 2009)	(Gardner -Gee and Beggs 2010; Towns 2002)

Beech forest: photograph shows bellbird feeding on honeydew (by Alan Leifting, with permission)

Kānuka scrub: photograph shows a moth feeding on honeydew (photo: RGG)

Coastal forest: photograph shows a gecko feeding on honeydew (photo RGG)

Coelostomidia zealandica honeydew in coastal forest (Towns, 2002), and only insects utilising *C. wairoensis* honeydew regularly in kānuka scrub (Gardner-Gee and Beggs, 2009, 2010).

From a global perspective, these communities are unusual, because ants, which often dominate honeydew resources elsewhere, play minor ecological roles in these forests (Gardner-Gee, R, unpublished data). Ants are found in all three forest communities (Ward, 2009). Some ant species such as *Prolasius advena*, *Monomorium* spp., and *Huberia* spp. feed on honeydew, but they do not seem to monopolize the resource, and the same species are wide spread in the absence of honeydew (pers. comm. Ward D.).

Despite the fact that New Zealand honeydew communities are distinctive and relatively well studied, the chemical composition of honeydew from these forests is poorly characterized. The sugar composition of honeydew from two *Ultracoelostoma* species (Coelostomidiidae: Homoptera) has been analyzed, showing a small proportion of glucose and larger but more variable proportions of fructose, sucrose, and unidentified oligosaccharides (Grant and Beggs, 1989; Beggs et al., 2005). There have been no analyses of *Coelostomidia* honeydew.

In this study, we analyzed the composition of honeydews from both genera of coelostomidiid scale insects that occur in New Zealand, collecting samples in three New Zealand honeydew consumer communities. We collected honeydew from *Coelostomidia zealandica, C. wairoensis*, *and Ultracoelostoma* sp. We used recently developed TMS- and MCF- derivatization methods (Villas-Bôas et al., 2006; Smart et al., 2010) followed by highly sensitive GC-MS detection technique to provide the first detailed account of these ecologically important honeydews.

We asked three questions: 1. Do the honeydews contain distinctive compounds that distinguish them from previously known honeydews, especially the ant-dominated honeydews? 2. Are the honeydews from the three New Zealand communities chemically distinct from each other? 3. Are there important site or host tree influences on honeydew composition?

Methods and Materials

Collection Methods and Sites The standing crop of honeydew was collected in 2008 from five sites in New Zealand, namely Karamatura Valley (−37.004051°, 174.556554°), Korapuki Island (−36.657787°, 175.849492°), Pelorus Bridge (−41.162914°, 172.819921°), Rotoiti (−41.832856°, 172.818879°), and Rotoroa (−41.909007°, 172.654177°), with the scale insect-host-site combinations described in Table 2. The standing crop represents the honeydew droplets hanging from the anal filaments and exposed to the environment. Since honeydew is constantly excreted by scale insects and removed by consumers, different droplets are exposed to the environment for different time periods and thus have undergone different amounts of chemical conversions induced by bacteria and yeasts associated with the sugar-rich environment. To obtain homogeneity and reduce variability due to droplet exposure time, each sample comprised numerous pooled droplets of honeydew from several scale insects on one tree. *Ultracoelostoma* spp. includes *Ultracoelostoma assimile* and *U. brittini*, which are morphologically indistinguishable in the field, exhibit no differences in host usage, and have been reported previously at the collection sites (Ball and Armstrong, 2007). At each site, at least six trees were sampled and 50 μl of honeydew were collected from each tree into microcapillary tubes. Samples were transported on ice at 4°C and were stored at −80°C until analysis.

Chemicals The methanol, sodium hydroxide, chloroform, and sodium sulfate were analytical grade and purchased from different suppliers (Smart et al., 2010). The derivatization reagents methylchloroformate (MCF), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), methoxylamine hydrochloride, and pyridine as well as the isotope-labeled internal standard L-alanine-2,3,3,3-d_4 and D-ribitol were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Sample Preparation Samples for analysis of sugars and derivatives were diluted 50-fold before mixing 10 μl of the diluted sample with 10 μl of the internal standard D-ribitol

Table 2 Sampling overview

	Ultracoelostoma spp.		*Coelostomidia wairoensis*		*Coelostomidia Zealandica*	
Host Number of trees sampled/site	Black beech *N*=6	Hard beech *N*=6	Red beech *N*=6	Kānuka *N*=6	Ngaio *N*=6	Karo *N*=1
Collection date	29–31 Jan 2008			11 Feb 2008	25 Feb 2008	25 Feb 2008
Collection site/s	Rotoiti (*N*=12) and Rotoroa (Nelson Lakes), Pelorus Bridge, (Marlborough)	Rotoroa (Nelson Lakes)	Rotoiti (Nelson Lakes)	Karamatura Valley (Auckland)	Korapuki Island (Coromandel)	Korapuki Island (Coromandel)

(10 μM). Samples were freeze-dried before derivitization, because drying with a speed vacuum apparatus caramelized them. Samples for analysis of amino and non-amino organic acids were prepared by combining 10 μl of undiluted honeydew sample with 10 μl of the internal standard D_4-alanine (10 mM) and 180 μl of 1 M NaOH. The alkalinized samples then were derivatized.

Sample Derivatizations The trimethylsilyl (TMS) derivatization for analysis of sugars and derivatives was based on the optimized protocol described by Villas-Bôas et al. (2006). In summary, the dried samples were resuspended in 80 μl of methoxyamine hydrochloride solution in pyridine (2 g/100 ml) and incubated in a domestic microwave oven for 2.8 min, with multimode irradiation set to 400 W and 30% of exit power. MSTFA (80 μl) then was added to each sample followed by 3.0 min incubation in a microwave oven as described above.

The MCF derivatization for analysis of amino and non-amino organic acids was performed according to Villas-Bôas et al. (2003). In summary, alkaline samples were mixed with 34 μl of pyridine and 167 μl of methanol before adding 20 μl of MCF and vigorously mixing for 30 s. An additional 20 μl of MCF were added to the reaction mixture followed by vigorous mixing for 30 s. To separate MCF derivatives from the reactive mixture, 400 μl of chloroform were added with vigorous mixing for 10 s, followed by the addition of 400 μl of sodium bicarbonate (50 mM) and vigorous mixing for 10 s. The upper aqueous layer was discarded, and the chloroform phase was analyzed with GC-MS.

GC-MS Analysis and Compound Identification GC-MS analysis was performed with as Agilent GC-MS system 6,890 gas chromatograph coupled to an Agilent 5,975 quadrupole mass selective detector on electron impact (EI) mode operated at 70 eV. The column used for all analyses was a ZB1701 (Zebron, Phenomenex), 30 m×250 μm i.d.× 0.15 μm film thickness. The MS was operated in scan mode (start after 4.5 min, mass range 40–650 a.m.u. at 0.15 s/scan). The parameters for separation and analysis of TMS and MCF derivatives are described in Villas-Bôas et al. (2006) and Smart et al. (2010), respectively. For compounds forming more than one major derivative, the most intense peak was selected for quantification. We used the Automated Mass Spectral Deconvolution and Identification System (AMDIS) to identify compounds present in each sample based on mass spectra and retention times against our in-house MS library of spectra. AMDIS is software freely distributed by the National Institute of Standards and Technology (http://www.amdis.net/) and has been widely applied to metabolite analysis. For more details on our library see Smart et al. (2010).

Data Analysis The TMS (sugars and derivatives) and MCF (amino and non-amino organic acids) data were treated separately throughout the analysis as they are two independent subsets, comprising relative abundance values of each compound detected in relation to the concentration of an internal standard added to the samples (D-ribitol or d_4-alanine). Each data set was standardized to binary presence-absence data for compositional analysis. Multi-dimensional scaling (MDS) was used on a binary distance matrix for comparing each dataset to variables such as scale insect species, host tree (within *Ultracoelostoma* spp.) at Rotoiti, and site (within *Ultracoelostoma* spp.) for black beech (*N. solandri*) host. Multiple sample sets of black beech were available from Rotoiti, allowing us to examine environmental differences by testing variability between two sets of samples collected from the same location. A non-parametric test of variance, Adonis, was used on the binary distance matrices for difference comparisons. Host differences at single sites and site differences of single hosts were compared across the chemical profiles of *Ultracoelostoma* spp. honeydew. All analyses were carried out in R (version 2.11.1) with additional packages Vegan and MASS, downloaded from CRAN servers (www.cran.r-project.org).

Results

Composition Seventy seven distinct compounds were detected in honeydew, comprised of 36 TMS derivatives (carbohydrates) and 41 MCF derivatives (amino- and non-amino organic acids). We identified 71% of the compounds, but the rest remained unidentified. Of those identified, 38% have not been reported previously associated with phloem-based honeydews.

Honeydews produced by *Ultracoelostoma* and *Coelostomidia* species contained a complex array of sugars and sugar-related compounds. The disaccharide sucrose was present in all honeydews sampled, as was the monosaccharide D-fructose and an unknown compound suspected to be 1-monooctadecanoylglycerol. D-glucose, maltose, meso-inositol, and sorbitol also were found in most samples. An additional 19 sugars and 9 other carbohydrate compounds were detected in some samples (Table 3).

Seven amino acids were detected, along with 34 other organic acids (Table 4). Among the amino acids, valine, leucine, isoleucine, proline, phenylalanine, glutamate, and tyrosine were detected in some samples. Of these, proline was most consistently present in honeydews from each species, but was not found in those produced by *C. zealandica* on karo (*Pittosporum crassifolium*). Several fatty acids including stearic (C18:0), oleic (C18:1n-9), palmitic (C16:0), levulinic (C5 keto acid), behenic (C22:0), and arachidic (C20:0) acid also were detected.

Table 3 Low molecular weight sugars and other metabolites detected in honeydew samples using trimethylsilyl (TMS) derivatization and GC-MS analysis. Significant *P*-values computed among the honeydews of the different species are indicated

Elution time (min)	Name of compound	% of samples						*P*-values
		Ultracoelostoma spp.			*Coelostomidia* spp.			
		Black beech (*N*=18)	Hard beech (*N*=6)	Red beech (*N*=6)	Kānuka (*N*=6)	Ngaio (*N*=6)	Karo (*N*=1)	
8.57	Lactic acid	33	50	50	67	33	100	
11.89	Glycerol[a]	0	0	0	0	17	100	**
13.17	Phosphate	0	0	0	0	17	0	
18.77	D-Fructose	100	100	100	100	100	100	**
19.23	D-Galactose	0	0	0	67	0	0	**
19.37	D-Glucose	100	100	100	83	83	100	**
19.65	D-Mannitol	0	0	0	0	100	100	**
19.85	Sorbitol	22	17	33	50	100	100	**
21.78	D-Chiro-inositol[a]	50	83	83	0	0	0	
22.67	L-Ascorbic acid (suspected)[b]	6	17	0	0	0	0	
22.93	Meso-inositol	94	83	100	100	100	0	**
30.02	Disaccharide Unknown 1	67	83	83	0	33	0	
31.29	Sucrose	100	100	100	100	100	100	
33.24	Disaccharide Unknown 2	0	0	0	17	100	100	**
33.35	Maltose	61	67	83	17	100	100	**
33.95	Disaccharide Unknown 3	0	0	0	67	67	0	**
34.14	Disaccharide Unknown 4	6	0	0	17	0	0	
34.51	Disaccharide Unknown 5	6	17	0	67	83	100	**
35.27	1-monooctadecanoylglycerol (suspected)[a b]	100	100	100	100	100	100	
35.46	Disaccharide alcohol Unknown 6	0	0	0	0	50	0	**
36.09	Unknown 7	17	17	17	0	17	0	
36.72	Melibiose (suspected)[c]	0	0	0	33	0	0	**
37.09	Disaccharide Unknown 8	0	0	0	0	100	0	**
38.95	Galactinol (suspected)[b]	17	17	17	50	0	0	
45.36	Alkaloid Unknown 9 (suspected)[b]	0	0	0	83	0	0	**
45.81	Trisaccharide Pyranoside Unknown 10	61	67	83	0	0	0	**
45.96	Trisaccharide Unknown 11	78	83	83	17	0	0	**
46.07	Trisaccharide Unknown 12	6	0	17	83	0	0	
46.92	Raffinose	89	83	100	100	0	0	**
47.11	Trisaccharide Pyranoside Unknown 13	6	17	17	0	0	0	
47.91	Trisaccharide Pyranoside Unknown 14	100	100	100	0	0	0	**
48.48	Melezitose	6	0	0	100	0	100	**
50.19	Trisaccharide Unknown 15	6	0	0	0	0	0	
51.08	Trisaccharide Unknown 16	6	0	0	0	0	0	
51.52	Maltotriose (suspected)[a b]	0	0	0	17	0	0	
52.77	Trisaccharide Unknown 17	11	50	0	0	0	0	

** = $P<0.001$, determined by three way ANOVA

[a] = first record of the compound in phloem honeydew

[b] = determined by similarity of mass spectra against commercial MS library.

[c] = determined by alignment of retention times to a set reference

Comparison of Honeydews Site did not have an effect on honeydew composition, with no differences observed in the MDS plots for the *Ultracoelostoma* spp. honeydews collected from black beech at the three sites (Map 1). Most

Table 4 Amino (in bold) and non-amino organic acids detected in honeydew samples using MCF derivatization and GC-MS detection. Significant *P*-values computed between the honeydews of the different species are indicated

Elution time (min)	Name of compound	% of samples						*P*-values
		Ultracoelostoma spp.			*Coelostomidia* spp.			
		Black beech (*N*=18)	Hard beech (*N*=6)	Red beech (*N*=6)	Kānuka (*N*=6)	Ngaio (*N*=6)	Karo (*N*=1)	
5.75	2-oxovalerate[a]	28	0	17	0	33	0	
6.15	Malonate	94	100	100	83	100	100	**
6.32	Oxalate[b]	11	33	17	83	67	100	
6.35	(3S)-3-Methyl-2-oxopentanoate[a]	33	67	33	0	0	0	
6.56	Caproate[a]	6	0	0	0	50	0	**
7.16	Unknown 18	100	100	100	100	100	100	
7.31	Ethyl-2-hydroxypentanoate (Suspected)[a b]	17	0	0	0	0	0	
7.53	Fumarate	100	100	100	67	67	0	**
8.04	Succinate	100	100	100	83	100	100	**
8.18	Lactate	100	100	100	100	100	100	**
8.20	4-methyl-2-oxopentanoate (Suspected)[a b]	83	100	100	0	0	0	**
8.21	Malate	11	0	0	0	0	0	
9.25	Unknown 19	100	100	100	83	100	100	
9.28	Itaconate[a]	6	0	0	33	17	0	
9.72	Oxaloacetate	11	17	0	0	0	0	
9.73	Levulinate[a]	6	0	0	0	17	0	
10.38	Benzoate[a]	6	17	0	0	0	0	
11.17	Unknown 20	11	0	0	100	0	0	
11.52	4-methylbenzoate (Suspected)[a b]	6	0	0	0	0	0	
11.75	Glycerate	6	0	17	0	0	0	
11.83	3-hydroxyoctanoate[a]	11	0	67	0	17	0	
12.35	Valine	6	0	0	17	0	0	
13.66	2-oxoglutarate	6	0	0	0	67	0	**
13.79	Leucine	11	17	17	0	0	0	
13.86	Isoleucine	22	17	17	17	0	0	
14.71	Proline	33	33	33	33	17	0	
15.19	Oxaloacetate	6	0	33	0	0	0	
15.71	Unknown 21	56	83	100	100	83	100	
16.37	Citrate	39	100	100	100	100	100	**
18.19	Hydroxybenzoate[a]	6	0	17	0	0	0	
18.40	Glutamate	6	0	0	33	0	0	
18.45	Isocitrate	6	0	0	0	17	0	
20.68	Phenylalanine	6	0	0	17	0	0	
22.52	4-Hydroxy-3-methoxybenzoate (Vanillate)[a]	6	0	0	0	0	100	**
23.08	Hexadecanoate (Palmitate)[b]	6	0	0	83	50	0	
25.42	(E)-9-Octadecenoate (Oleate)[a b]	6	0	0	17	17	0	
25.66	Octadecanoate (Stearate)[a b]	6	0	0	100	50	100	**
28.85	Eichosanoate (Arachnidate)[a b]	6	0	0	83	0	0	**
29.58	Tyrosine	6	0	0	17	0	0	
31.93	Docosanoate (Behenoate)[a b]	6	0	0	33	0	0	
38.18	Cholesta-3,5-diene (Suspected)[a b]	6	0	0	0	17	0	

** = $P<0.001$, determined by three way ANOVA

[a] = first record of the compound in phloem honeydew

[b] = determined by similarity of mass spectra against a commercial MS library

variability in the data was captured within the first two dimensions [TMS: Eigen values (Eig.) 0.6860109 0.3205074; MCF: Eig. 0.6747290 0.3029696; plots a, d Fig. 1]. Host also did not have a discernible effect on honeydew composition, since honeydew from *Ultracoelostoma* spp. collected from red (*N. fusca*) and black (*N. solandri*) beech (both at Rotoiti) did not show any clear patterns in the MDS plots, and the first two dimensions captured most of the variability (TMS: Eig. 0.5404770 0.2291629; MCF: Eig 0.6402101 0.2265920, see plots e, f Fig. 1). In order to compare the three scale insect species, we pooled the 36 *Ultracoelostoma* samples and compared the composition to that of the honeydews of *C. wairoensis* and *C. zealandica*. This analysis of the MCF and TMS derivatives revealed significant differences among the three species, and indicated that each scale insect species had a distinctive honeydew composition (MCF: Eig 3.425742 1.050942; TMS: Eig 3.971345 1.881466, see plots a, b Fig. 1).

Multiple sample sets of honeydew from black beech collected at a single site at Rotoiti were tested bi-directionally (with pooled host variances and site variances) using non-parametric test ADONIS, and no significant differences were found in the TMS or MCF composition, suggesting minimal variation due to collection effects (see Table 5 for *P*-values). This also suggests that environmental variability due to differential environmental influence on each individual drop is diminished as the samples are pooled. Red beech and black beech from Rotoiti had a small significant difference in the TMS residues (P=0.004), while no difference was observed for the MCF residues. Neither TMS nor MCF residues showed any significant compositional differences between hard beech (*N. truncata*) and black beech samples from Rotoiti. TMS residues from black beech samples from Pelorus, Rotoiti and Rotoroa showed a small but significant difference (P=0.001), while the MCF residues had smaller differences (P=0.041).

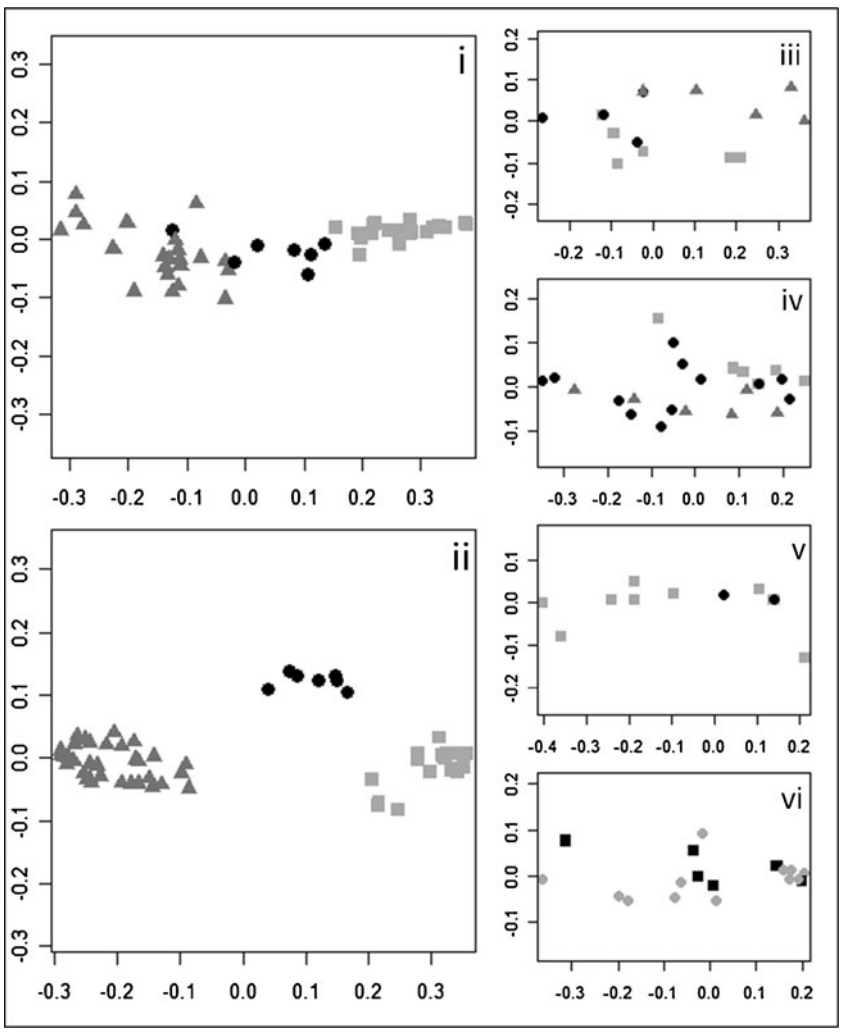


Fig. 1 Multidimensional scaling plots comparing honeydew composition. **i**, amino and non-amino organic acid composition (MCF derivatives) and, **ii**, carbohydrate composition (TMS derivatives) between the three scale insect species, namely *Ultracoelostoma* spp. (*triangle*), *Coelostomidia zealandica* (*circles*) and *C. wairoensis* (squares); amino and non-amino organic acid composition between sites (**iii**) and hosts (**v**) and carbohydrate composition between the sites (**iv**) and hosts (**vi**). Hosts of *Ultracoelostoma* spp. were black beech (*squares*) and red beech (*circles*) at site Rotoiti; and the three sites of *Ultracoelostoma* spp. were Rotoiti (*circles*), Pelorus Bridge (*triangles*) and Rotoroa (*squares*) with host black beech

Table 5 Summary of Adonis results to test variance within hosts (BB: Black beech, RB: Red beech and HB: Hard beech) at one site, within sites (Pel: Pelorus, Roi: Rotoiti and Roa: Rotoroa) with single host and within multiple sample sets at single site (BB, BB) or with single host (Roi, Roi). * = $P<0.005$, ** = $P<0.001$

Data set	Subset	Comparison	n each	F model	R^2	*P*-value	Significance
TMS	Ultracoelostoma$Rotoiti	BB/RB	$N=6$	9.958	0.499	0.004	**
TMS	Ultracoelostoma$Rotoiti	BB/BB	$N=6$	1.525	0.132	0.239	
MCF	Ultracoelostoma$Rotoiti	BB/RB	$N=6$	1.021	0.093	0.419	
MCF	Ultracoelostoma$Rotoiti	BB/BB	$N=6$	1.897	0.160	0.129	
TMS	Ultracoelostoma$Rotoroa	HB/BB	$N=6$	1.753	0.149	0.178	
MCF	Ultracoelostoma$Rotoroa	HB/BB	$N=6$	1.029	0.093	0.414	
TMS	Ultracoelostoma$Blackbeech	Pel/Roi/Roa	$N=6$ ($N=12$ for Roi)	7.724	0.424	0.001	**
TMS	Ultracoelostoma$Blackbeech	Roi/Roi	$N=6$	1.525	0.132	0.251	
MCF	Ultracoelostoma$Blackbeech	Pel/Roi/Roa	$N=6$ ($N=12$ for Roi)	2.009	0.160	0.041	*
MCF	Ultracoelostoma$Blackbeech	Roi/Roi	$N=6$	1.897	0.159	0.128	

Discussion

Over 75 distinct compounds were detected from honeydew, of which more than a third have not been reported previously associated with phloem-based honeydew from any insect (Tables 3 and 4). Blüthgen's work on honeydews of ant-attended homopteran insects reported only 25 compounds by using high-performance liquid chromatography (HPLC) (Blüthgen et al., 2004). Chemical analysis of honeydew is not an easy task due to the high sugar content of the samples, which severely interferes with the analysis of other, non-sugar components. However, by using a relatively novel derivatization method designed for metabolome analysis, MCF, we identified 16 non-sugar metabolites by using only 10 μl of sample (Villas-Bôas et al., 2011). This could be accomplished because the MCF derivatization minimizes the interference of sugars by extracting the derivatized analytes with chloroform, and because the GC-MS system is highly sensitive (Villas-Bôas et al., 2011). MCF cannot be used to derivatize sugars and related compounds. However, the TMS method described in Villas-Bôas et al. (2006) is optimized to simultaneously analyze over 53 different sugars and derivatives (monosaccharides, disaccharides, trisaccharides, sugar alcohols, and amino sugars). This method has been employed here for honeydew analyses for the first time. This pushes the boundaries of honeydew compositional analyses by reporting many additional compounds such as glycerols, sugar alcohols, fatty acids, dienes, alkaloids, and other organic acids along with the well described sugars and amino acids.

The reported carbohydrate composition of honeydew usually is limited to a dozen or so well-known sugars (Hendrix et al., 1992), some of which were detected in our analysis (Table 6). Honeydew carbohydrate composition varies among homopteran species and among host species (Hendrix et al., 1992; Fischer and Shingleton, 2001), but usually consists of the disaccharide sucrose, its component monosaccharide sugars (fructose and glucose), and one or more oligosaccharides (Byrne et al., 2003) (Table 6). In our study, we detected sucrose in all honeydew samples, suggesting that scale insects are not completely utilizing all of the plant sugars. They are instead venting some of it directly to the outside, by-passing most of the midgut (Foldi, 1990) where sucrase activity is maximal

Table 6 List of known honeydew amino acids and carbohydrates. Data from this study and published papers (Blüthgen et al., 2004; Byrne et al., 2003; Douglas, 1998; Fischer and Shingleton, 2001; Grant and Beggs, 1989; Gray, 1952; Hendrix et al., 1992; Lamb, 1959; Völkl et al., 1999; Yao and Akimoto, 2001). Essential and non-essential amino acid status notation based on Douglas, 1998 and Chapman, 1998. Compounds in bold were reported in the present study

Essential amino acids	Non-essential amino acids	Carbohydrates
Arginine	α-aminoapidic acid	Xylose
Histidine	Alanine	Fructose
Iso-leucine	Asparagine	Galactose
Leucine	Aspartic acid	Glucose
Lysine	β-amino butyric acid	Maltose
Methionine	Citrulline	Melibiose
Phenylalanine	Cystine	Sucrose
Threonine	Glutamate	Trehalose
Typtophan	Glutamine	Trehalulose
Valine	Glutathione	Turanose
	Glycine	Bismisiose
	o-phosphoethanolaminne	Erlose
	Proline	Raffinose
	Serine	Melezitose
	Tyrosine	Maltotriose
		Stigmatriose
		Stigmapentose
		Stachyose
		Stigmatetrose

(Karley et al., 2005). We also detected the trisaccharide raffinose, which is one of the three main types of sugars translocated in the phloem and has been detected occasionally in honeydews (Byrne et al., 2003). Components of sucrose and raffinose (galactose, fructose, and glucose) commonly occurred in our samples, along with maltose (a disaccharide composed of two glucose units). A few other unknown disaccharides were present in many of the samples.

Polyols such as meso-inositol, sorbitol, mannitol, and galactinol were detected in our samples. Mannitol, which was present only in *C. zealandica* honeydew, and sorbitol , are thought to provide thermo- and osmoprotection in aphids and whiteflies, respectively (Hendrix and Salvucci, 1998). 1-Monooctadecanoylglycerol is a lipid precursor that has been detected previously in sugarcane plants (Glassop et al., 2007) but not in honeydews, and it may be plant-derived.

The remaining sugars and compounds that we detected in the honeydews could be scale insect, host plant, or microbially derived, from the microbes that colonize the insects or the droplets of honeydew. For example, it is thought that phloem-feeding insects reduce the sucrose content of ingested phloem sap by polymerization processes that yield oligosaccharides, thus regulating gut osmotic pressure (Hendrix et al., 1992; Byrne et al., 2003; Douglas, 2006). Nine other trisaccharides were present, but we were unable to confirm their identities.

Insects depend on external sources to acquire essential amino acids (Chapman, 1998), but the requirements vary among species and are not known for scale insects. We found seven amino acids in scale insect honeydew (Table 6). Since scale insects are unable to synthesize amino acids, these were either derived from the host plant, or synthesized from plant precursors with the aid of bacterial inhabitants (Douglas, 1998). For example, the sharpshooter *Homalodisca coagulata* contains the bacterial symbiont *Sulcia muelleri*, which can synthesize proline (McCutcheon and Moran, 2007). Bacterial symbionts closely related to *S. muelleri* have been found previously in *C. wairoensis* (Dhami MK, unpublished data). Chemical analysis of phloem composition would allow us to determine whether phloem is the source of these amino acids.

A number of organic acids including citric, malic, succinic, lactic, and fumaric acids also were detected in our samples and have been reported previously from honeydew of aphids and mealybugs (Gray, 1952; Lamb, 1959; Hussain et al., 1974). These compounds may play a role in altering the pH of honeydew, which may result in some consumers being deterred or attracted to honeydew.

The honeydews reported here contained many compounds not detected in earlier research. Technological advances are primarily responsible for these differences, as our methods were able to detect and identify many more compounds than previously possible. Even so, despite the high resolution of the technique, 27% of detected compounds were categorized as unknown, due to a lack of corresponding advances in compound identification.

The main influence on honeydew composition is likely to be the species of scale insect, consistent with honeydew from aphids (Völkl et al., 1999). Site and host did influence amino- and non-amino organic acid composition. Closer sites (Rotoiti and Rotoroa, map1) had more similar compositions, compared to the more distant site (Pelorus Bridge), probably because environmental factors such as local climate influence the biochemistry of the forest ecosystem. Two species of host also had a small significant difference on the amino acid composition of *Ultracoelostoma* spp. honeydew. However, both site and host influences were minor compared to the influence of scale insect species (Fig. 1). Each of the three New Zealand honeydew communities may, therefore, be based upon chemically distinct honeydew because it is produced by a different scale insect species in each system. Some components of honeydew potentially could attract or deter different consumers. For example, melezitose is proposed as an ant attractant in some studies (Völkl et al., 1999; Fischer and Shingleton, 2001; Yao and Akimoto, 2001), although it does not act as an ant attractant in all honeydews (Romeis and Wäckers, 2000; Blüthgen and Fiedler, 2004). We found melezitose in all *C. wairoensis* honeydew collected from kanuka (*Kunzea ericoides*) (Table 3), however, only a few ants were observed feeding on *C. wairoensis* honeydew in a recent survey (Gardner-Gee, unpublished thesis), which suggests that melezitose may not have an ant-attraction role in this system. Nevertheless, a wide range of new compounds were detected in this study, and some of these compounds may have an influence on chemo-sensitive consumers, such as wasps and other parasitoids (Wäckers, 2001). Further testing of specific honeydew compounds paired with consumer groups, as has been done for EFNs and nectar (González-Teuber and Heil, 2009a, b), is needed to fully unravel these relationships.

Honeydew is composed of a complex variety of compounds, some of which have been reported here for the first time. Our study highlights the incomplete nature of our knowledge of honeydew composition and the unexplored potential of honeydew as a source of novel and rare compounds. Many of the detected compounds potentially could influence consumer communities, but at this stage it is difficult to hypothesize their roles. Comparison of scale insect species has shown that they have a significant influence on the chemical composition of their respective honeydews, which may explain the characteristic consumer communities associated with them.

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At the same time, reproductive queens must produce signals that regulate reproductive development and behavior of other colony members (Keller and Nonacs, 1993). Elucidation of the mechanisms that underlie the communication of individual reproductive potential and status is critical for understanding the organization of ant societies.

A key question regarding these mechanisms is what compounds are used to mediate the interactions between queens and workers? Studies suggest that cuticular hydrocarbons play a central role in nestmate discrimination, queen attraction, and regulation of reproduction in many ants, bees, wasps, and termites (Monnin, 2006; Le Conte and Hefetz, 2008; Liebig et al., 2009; Peeters and Liebig, 2009; Weil et al., 2009; van Zweden and D'Ettorre, 2010). The hydrocarbon characteristic of reproductive queens in the ant *Pachycondyla inversa*, for example, elicits a strong antennal response in electro-antennograms of workers, thus suggesting a sensory bias to this compound (D'Ettorre et al., 2004). In the ant *Aphaenogaster cockerelli*, workers with highly active ovaries produce cuticular hydrocarbons identical to those of reproductive queens (Smith et al., 2008); such workers are attacked by nestmates, presumably preventing them from reproducing. These attacks can be triggered in laboratory assays by the alkane *n*-pentacosane, the main component of the hydrocarbon profile of reproductive queens (Smith et al., 2008). Other direct evidence for an important role for hydrocarbon profiles in the regulation of ant reproduction comes from egg discrimination experiments in *Camponotus floridanus*. Reproductive queens produce a hydrocarbon profile that contains roughly twice the number of components as the profiles of non-reproductive queens (Endler et al., 2006). These reproductive queen hydrocarbon profiles also are present on queen-laid eggs, but not on worker-laid eggs, allowing workers to recognize and eliminate the latter (Endler et al., 2004). Finally, a primer effect of a major hydrocarbon compound produced by fertile queens of the ant *Lasius niger* has been demonstrated through reduced ovarian development of workers exposed to this compound compared to controls (Holman et al., 2010).

Given the widespread association between cuticular hydrocarbon profiles and reproductive status in eusocial insects, and the direct evidence for these compounds as fertility signals, it is especially interesting to find and explain possible exceptions to this pattern. In the red imported fire ant, *Solenopsis invicta*, the role of cuticular hydrocarbons has been studied only in the context of nestmate discrimination, with experimental transfer of hydrocarbons having no effect, thus suggesting that these compounds are not involved in this behavior (Anderson and Vander-Meer, 2001). Because cuticular hydrocarbons are involved in nestmate discrimination in many other social insect species (van Zweden and D'Ettorre, 2010), their lack of use in nestmate discrimination for *S. invicta* suggests that they also may not be involved in fertility signaling. In fact, hydrocarbon patterns for the postpharyngeal glands of queens did not change over the course of 10 days following mating and initiation of oogenesis (Vander Meer et al., 1982), a period during which such signaling to potential reproductive rivals or worker nestmates should be important.

Previous studies have shown that fully reproductive *S. invicta* queens produce a pheromone that inhibits winged, virgin queens from shedding wings (dealating) and developing ovaries (Fletcher and Blum, 1981; Vargo, 1998); workers are not affected in this way, because they have rudimentary ovaries and cannot lay eggs. The inhibitory pheromone(s) apparently originates from the venom sac and the postpharyngeal glands (Vargo and Hulsey, 2000), but the components are yet to be identified. The venom sac also has been shown to be the source of large amounts of venom alkaloids (Brand et al., 1972, 1973) and of pyranones. The latter are attractive to workers (Rocca et al., 1983a,b) but do not inhibit the reproductive development of winged queens (Glancey et al., 1984). In workers, the most abundant components of the alkaloids are *trans*-2-methyl-6-(*cis*-4′-*n*-tridecenyl) piperidine (*trans*-$C_{13:1}$), *trans*-2-methyl-6-*n*-tridecylpiperidine (*trans*-C_{13}), *trans*-2-methyl-6-(*cis*-6′-*n*-pentadecenyl) piperidine (*trans*-$C_{15:1}$), and *trans*-2-methyl-6-*n*-pentadecylpiperidine (C_{15}). In alate queens, the venom constituents are different, with the most abundant component being *cis*-2-methyl-6-*n*-undecylpiperidine (*cis*-C_{11}) (Brand et al., 1973). While workers inject venom directly into other animals for defense and predation, or spray it throughout the nest environment, presumably for protection against microbial pathogens, queens apply it over eggs as they are laid. It has been suggested that this behavior protects eggs from entomopathogenic fungi (Vander-Meer and Morel, 1995; Tschinkel, 2006). However, this behavior also raises the possibility that venom alkaloids on the surface of eggs may play a role in advertising the presence and fertility status of queens, as do hydrocarbons in other social insects.

An added element of complexity in the pheromonal regulation of reproduction and social organization in *S. invicta* comes from the occurrence of two social forms: monogyne, in which colonies contain a single queen, and polygyne, in which colonies contain multiple queens. The two social forms differ in several ways. For example, monogyne colonies are initiated by a single queen who raises her first brood by using body reserves, while polygyne colonies are initiated by budding, a process in which queens and workers leave the colony to initiate a new colony nearby (see Ross and Keller, 1995). Polymorphism in social organization is associated with allelic variation at the gene *Gp-9*; in the USA, monogyne colonies contain only inhabitants bearing the *B* allele, while polygyne colonies also include inhabitants bearing the *b*

allele (Gotzek and Ross, 2007). Monogyne workers (all of which are *BB* homozygotes) accept a single *BB* replacement reproductive queen if their colony has been queenless for several days, but they do not tolerate queens bearing the *b* allele. Alternatively, polygyne workers (a mix of individuals with and without the *b* allele) accept multiple reproductive queens bearing the *b* allele, but do not tolerate *BB* queens (Ross and Keller, 1998, 2002). This genotype-specific pattern of aggression or tolerance of queens by workers extends to nestmate non-reproductive queens in polygyne colonies; worker aggression toward young *BB* queens escalates as these queens approach two weeks of adult age, coincident with attainment of sexual maturity and reproductive competence (Keller and Ross, 1993a, 1999; Brent and Vargo, 2003). Ultimately, all sexually mature *BB* queens in polygyne colonies are executed or leave the nest on mating flights (Keller and Ross, 1999). Importantly, worker aggression toward *BB* queens in polygyne colonies is induced by a transferable queen signal on the cuticle (Keller and Ross, 1998). While production of this signal is related to weight and tendency to initiate egg-laying in non-reproductive queens, it is not related to fecundity in reproductive queens (Keller and Ross, 1993a, 1999; Ross and Keller, 1998). This has led to the suggestion that at least two distinct queen-produced pheromones are involved in regulation of colony queen number and identity in *S. invicta*: one communicating *Gp-9* genotype and the other communicating actual or potential reproductive status (Keller and Ross, 1999, Gotzek and Ross, 2007).

Here, we compared venom alkaloid and cuticular hydrocarbon profiles of queens of different stages of sexual maturity, reproductive status, and *Gp-9* genotype, as well as workers, originating from monogyne and polygyne *S. invicta* colonies. If cuticular hydrocarbons do not encode any of the above-mentioned types of information, then the abundant piperidines, unique to the venom of this group of ants, may fill this role instead. Our goal was to detect associations between chemical profiles and reproductive state/genotype of different classes of colony members. Such associations may indicate a role for specific compounds as releaser or primer pheromones involved in communicating *Gp-9* genotype and/or fertility status to workers, roles essential to the regulation of colony social organization in fire ants.

Methods and Materials

Insects Large colonies of each social form of *Solenopsis invicta* were collected and reared in the laboratory under standard conditions (Ross, 1988). A total of 25 polygyne and 23 monogyne colonies were collected from Athens-Clarke Co. and Oglethorpe Co., Georgia, USA, respectively. Social organization of the polygyne colonies was determined by finding multiple reproductive (wingless, egg-laying) queens in each. Social organization of the monogyne colonies was determined by finding only a single reproductive queen in each. Social organization was subsequently confirmed for all colonies by genotyping several reproductive queens (polygyne colonies) or 20 adult workers (monogyne colonies) at the gene *Gp-9*, using horizontal starch gel electrophoresis coupled with amido black staining (DeHeer et al., 1999); all polygyne reproductive queens possessed the *Bb* genotype, while all monogyne workers possessed the *BB* genotype, as expected (Gotzek and Ross, 2007). Colonies were fed daily by alternating a high-protein diet (tuna/dog food/peanut butter mix) with a high-carbohydrate diet (assorted vegetables/granulated sugar mix). These diets were supplemented with crickets and/or mealworms on a twice-weekly basis.

Several hundred adult workers from individual laboratory colonies were collected by placing a glass vial upright in the foraging area of each rearing unit for several minutes, and allowing workers to fall into the vial; vials were placed briefly on dry ice, then stored at −80°C until extraction. Thirteen colonies of each social form were sampled in this way for determination of piperidine alkaloid profiles, while ten colonies of each form were sampled for determination of hydrocarbon profiles (see Table 1 for sample size information).

We collected queens at three different key stages of sexual and reproductive development. First, we collected 2d-old, sexually immature and non-reproductive, queens from polygyne colonies. These were obtained by transferring 1–6 newly emerged (callow) adult queens to small rearing units with several hundred worker brood and adults from the natal colony (see Table 1 for sample sizes). Forty-eight hours after transfer, queens were collected for chemical analysis. Second, we collected 14d-old queens from polygyne colonies. By this age, queens have undergone extensive weight gain and accumulation of energy reserves (fat and glycogen), characteristic of completion of maturation in *S. invicta* (Keller and Ross, 1993a, 1999; Brent and Vargo, 2003). Such queens are sexually mature, but typically embark on a mating flight and are inseminated before becoming reproductively active. Two to six newly emerged queens from each polygyne source colony were held individually in small rearing units, with worker brood and adults from the natal colony, for 14 d before being collected for analysis (see Table 1 for sample sizes). These queens were held individually, rather than in groups, because workers execute queens with a *Gp*-9 *BB* genotype when they attain sexual maturity in colonies that also contain queens with a *Bb* genotype (see Keller and Ross, 1998, 1999). This procedure also ensured that the reproductive development of young queens was not inhibited by the presence of other queens in the same unit. In the rearing

Table 1 Piperidine alkaloid and hydrocarbon composition of whole body surface extracts of *Solenopsis invicta*

Compound	MS fragments [*m/z* (relative%)][a]	Non-reproductive queens			Reproductive queens		Workers	
		2d-old	14d-old-*BB*[b]	14d-old-*Bb*[b]	*BB*[b]	*Bb*[b]	monogyne	polygyne
Piperidines		*N*=10,*n*=35[c]	*N*=6,*n*=8	*N*=11,*n*=27	*N*=7,*n*=7	*N*=13,*n*=13	*N*=13	*N*=13
cis-2-methyl-6-*n*-undecylpiperidine (*cis*-C_{11})	253 (M^+, 0.7), 252 (2.9), 238 (5.0), 98 (100)	54.4±7.0[d]	58.6±6.0	48.5±11.6	83.3±6.7	82.1±5.1	–[e]	–
trans-2-methyl-6-*n*-undecylpiperidine (*trans*-C_{11})	253 (M^+, 0.7), 252 (2.9), 238 (5.0), 98 (100)	29.7±4.8	28±4.0	31.5±7.1	3.2±2.8	2.4±3.0	1.4±0.9	0.7±0.2
Unidentified piperidine #1		1.2±1.2 (29)[f]	–	–	–	–	–	–
cis-2-methyl-6-(*cis*-4′-*n*-tridecenyl) piperidine (*cis*-$C_{13:1}$)	279 (M^+, 2.9), 264 (5.8), 180 (5.0), 124 (24.5), 111 (20.1), 98 (100)	1.3±0.9	2.5±1.1	3.8±1.7	5.3±3.0	4.2±1.3	–	–
cis-2-methyl-6-*n*-tridecylpiperidine (*cis*-C_{13})	281 (M^+, 0.7), 280 (2.1), 266 (2.9), 98 (100)	9.0±3.0	5.0±1.1	6.7±2.0	6.1±3.2	8.2±3.7	–	–
trans-2-methyl-6-(*cis*-4′-*n*-tridecenyl) piperidine (*trans*-$C_{13:1}$)	279 (M^+, 2.9), 264 (5.8), 180 (5.0), 124 (24.5), 111 (20.1), 98 (100)	0.5±0.8 (23)	0.8±0.5	1.6±1.5	–	–	27.0±4.9	41.6±4.5
Unidentified piperidine #2	279 (M^+, 0.5), 254 (1.5), 184 (6.0), 98 (100)	–	1.0±0.4	0.8±0.6 (24)	–	–	–	–
trans-2-methyl-6-*n*-tridecylpiperidine (*trans*-C_{13})	281 (M^+, 0.7), 280 (2.1), 266 (2.9), 98 (100)	3.9±2.0	3.0±1.0	5.4±3.0	–	–	14.6±1.9	11.6±1.7
Unidentified piperidine #3	305 (M^+, 3.2), 290 (3.0), 154 (4.7), 98 (100)	–	–	–	0.5±0.5	0.2±0.1	–	–
Unidentified piperidine #4	307 (M^+, 3.0), 292 (3.0), 154 (4.5), 98 (100)	–	0.3±0.2	0.5±0.5	0.4±0.3 (6)	0.3±0.2	–	–
cis-2-methyl-6-(*cis*-6′-*n*-pentadecenyl) piperidine (*cis*-$C_{15:1}$)	307 (M^+, 4.3), 292 (5.8), 208 (1.4), 154 (2.9), 98 (100)	–	–	–	0.6±1.1	0.1±0.0	–	–
cis-methyl-6-*n*-pentadecylpiperidine (*cis*-C_{15})	309 (M^+, 0.7), 38 (2.9), 294 (5.8), 98 (100)	–	0.1±0.1	0.3±0.2	0.6±0.7	0.5±0.8	–	–
trans-2-methyl-6-(*cis*-6′-*n*-pentadecenyl) piperidine (*trans*-$C_{15:1}$)	307 (M^+, 4.3), 292 (5.8), 208 (1.4), 154 (2.9), 98 (100)	–	0.4±0.2	0.6±0.8	–	–	43.0±2.1	35.7±4.7
trans-2-methyl-6-*n*-pentadecylpiperidine (*trans*-C_{15})	309 (M^+, 0.7), 38 (2.9), 294 (5.8), 98 (100)	–	0.2±0.1	0.3±0.2	–	–	13.0±2.5	8.9±1.8
trans-2-methyl-6-(*cis*-8′-*n*-heptadecenyl) piperidine (*trans*-$C_{17:1}$)	335 (M^+, 2.2), 334 (2.2), 320 (3.6), 236 (0.7), 98 (100)	–	–	–	–	–	1.0±0.5	1.4±2.3
Hydrocarbons		*N*=11,*n*=21[c]	*N*=10,*n*=20	*N*=14,*n*=24	*N*=12,*n*=12	*N*=10,*n*=18	*N*=10	*N*=10
Unsaturated								
[3][g] x-heptacosene ($C_{27:1}$)	378 (M^+, 20), 97 (100)	–	–	0.2±0.6 (7)	–	2.5±1.0	–	–
[18] x,y-nonacosadiene ($C_{29:2}$)	404 (M^+, 13), 96 (34)	–	–	0.1±0.4 (2)	–	2.6±0.5	–	–
[19] x-nonacosene ($C_{29:1}$)	406 (M^+,10), 97 (63)	–	0.1±0.2 (2)	0.4±0.5 (17)	0.1±0.2 (3)	4.4±1.4	–	–
[23] x,y-hentriacontadiene ($C_{31:2}$)	432 (M^+, 29), 96 (98)	<0.1 (2)	0.2±0.4 (11)	0.9±0.6 (23)	0.5±0.3 (11)	6.0±1.7	–	–
[24] x-hentriacontene ($C_{31:1}$)	434 (M^+,14), 97 (100)	–	0.1±0.1 (6)	0.4±0.4 (22)	0.2±0.2 (8)	2.3±1.0	–	–
Saturated								
[1] n-pentacosane (n-C_{25})	352 (M^+,3.9), 57 (100)	0.8±0.4	0.5±0.2	1.3±0.8	0.5±0.2	1.5±0.8	0.5±0.1	0.4±0.1

Table 1 (continued)

Compound	MS fragments [*m/z* (relative%)][a]	Non-reproductive queens			Reproductive queens		Workers	
		2d-old	14d-old-*BB*[b]	14d-old-*Bb*[b]	*BB*[b]	*Bb*[b]	monogyne	polygyne
[2] 12- and 13-methylhexacosane (12- and 13-MeC_{26})	365 (M^+-15, 4.2), 224 (4.2)/182(7.8), 210 (14)/196 (15.7)	1.3±0.2	1.5±0.2	1.3±0.3	1.7±0.6	1.0±0.3	1.1±0.5	0.6±0.2
[4] n-heptacosane	380 (M^+, 4.0), 57 (100)	3.5±0.9	4.5±1.4	7.7±2.4	7.4±2.4	12.4±5.2	17.5±3.5	22.6±3.1
[5] Unknown 1		–	0.3±0.6 (4)	0.1±0.4 (5)	1.5±1.2 (10)	<0.1±0.2 (1)	–	–
[6] Unknown 2		0.3±0.1	0.7±1.3 (13)	0.8±1.8 (20)	5.2±3.3	0.3±0.3 (13)	0.7±0.4	0.8±0.3
[7] 13-methylheptacosane (13-MeC_{27})	379 (M^+-15, 5.0) 224 (20.7)/196 (28.6)	29.9±3.4	31.5±2.7	29.6±3.5	21.9±3.4	17.6±8.4	22.6±1.9	20.1±1.9
[8] 5-methylheptacosane (5-MeC_{27})	337 (32.0)/85 (90.0)	1.1±0.1	1.0±0.4	0.9±0.3	2.0±0.5	0.9±0.2	0.6±0.1	0.5±0.0
[9] Unknown 3		0.1±0.1 (8)	0.4±0.9 (11)	0.4±1.0 (18)	1.7±0.9 (11)	<0.1±0.2 (2)	<0.1 (1)	<0.1 (3)
[10] 13,15-dimethylheptacosane (13,15-$DiMeC_{27}$)	239 (32.0), 210 (12.9), 196 (16.4)	18.1±1.9	19.7±1.6	17.3±1.7	17.3±2.1	11.9±1.8	13.0±1.7	11.2±1.6
[11] 3-methylheptacosane (3-MeC_{27})	365 (M^+-15, 52.1)/57 (100)	10.2±3.3	9.5±1.5	10.7±2.5	10.6±2.0	12.3±2.3	12.7±1.3	13.2±0.7
[12] 5,x-dimethylheptacosane (5,x-$DiMeC_{27}$)	393 (M^+-15, 4.3) 351 (11.4)/85(68.7)	1.1±0.3 (20)	1.1±0.2	1.0±0.2	1.1±0.5 (11)	0.8±0.1	0.6±0.1	0.5±0.1
[13] n-octacosane (n-C_{28})	394 (M^+, 2.1), 57 (100)	0.5±0.2 (20)	1.0±0.5	1.4±1.2 (23)	2.8±1.3	1.0±0.5 (16)	2.7±0.6	3.2±0.4
[14] 3,9- and 3,11-dimethylheptacosane (3,9- and 3,11 $DiMeC_{27}$)	379(10.7)/57 (100), 281(10.7)/155 (15), 253 (5.0)/183(5.0)	26.0±2.5	20.5±1.4	18.2±2.2	18.6±3.5	15.4±2.6	12.9±2.2	9.9±1.8
[15] 12-, 13-, and 14-methyloctacosane (12-, 13-, and 14-MeC_{28})	253 (2.1)/182 (5.7), 238 (5.0)/196 (7.1), 224 (12.9)/210 (12.1)	1.7±0.2	2.0±0.2	1.7±0.2	1.5±0.3	1.2±0.4 (19)	1.5±0.3	1.1±0.2
[16] 8,12-dimethyloctacosane (8,12-$DiMeC_{28}$)	323 (7.9)/127 (15.0), 253 (7.1)/197 (9.3)	1.5±0.2	1.3±0.2	1.1±0.2	1.2±0.2	1.0±0.3 (19)	0.8±0.3	0.5±0.2 (9)
[17] 14,16-dimethyloctacosane (14,16-$DiMeC_{28}$)	253 (12.1)/196 (8.6), 239 (12.9)/210 (10.7)	1.2±0.1	1.4±0.1	1.2±0.4	1.1±0.2	–	1.0±0.1	0.9±0.1
[20] n-nonacosane (n-C_{29})	408 (M^+, 3.6), 57 (100)	0.7±0.2	0.9±0.3	1.3±0.5	1.2±0.7	2.3±0.9	8.2±2.3	11.5±2.0
[21] 13-, and 15-methylnonacosane (13-, and 15-MeC_{29})	252 (10.7)/196 (16.4), 224 (12.9)	1.1±0.2	1.0±0.1	1.0±0.2	0.6±0.1	1.1±0.2	0.8±0.2	0.5±0.1
[22] 3-methylnonacosane (3-MeC_{29})	393 (M+-15, 42.9)/57 (100)	0.8±0.2	0.8±0.2	1.0±0.2	1.0±0.4	1.4±0.2	2.8±0.6	2.6±0.5

[a] Indicative mass spectrum fragments and their relative abundance; M^+ is the molecular ion

[b] *BB* and *Bb* are genotypes at the gene *Gp-9*

[c] Sample sizes for the analyses (*N* = number of colonies, *n* = number of individuals); for workers, several hundred (piperidine analysis) or 40 (hydrocarbon analysis) workers per colony were pooled for analysis

[d] Relative abundance, $\overline{X}$±SD

[e] Dashes indicate that only values of zero were obtained

[f] Numbers of non-zero values are shown in parentheses in cases where values of zero also were obtained

[g] Numbers in brackets refer to compounds in Fig. 5

units, some of the 14d-old queens dealated, a behavior associated with onset of oogenesis (Fletcher and Blum, 1981; Brent and Vargo, 2003); the identities of these queens were recorded. No 2d-old queens dealated. The third category of queens was reproductive. We collected one or two of these queens from polygyne laboratory colonies and the sole queen heading monogyne colonies (see Table 1). The mating status of the polygyne reproductive queens was unknown [approximately one-third of such queens remain unmated in invasive populations in the USA (Ross and Keller, 1995)], whereas all monogyne reproductive queens were assumed to have mated. All reproductive queens were collected after the colonies had been maintained in the laboratory for 1 month.

All collected queens were held in glass vials at −80°C, for several days to several weeks, pending chemical extraction. The *Gp-9* genotypes of all 14d-old queens were determined by starch gel electrophoresis following chemical extraction; 14d-old queens of both genotypes (*BB*, *Bb*) were included in the study (Table 1). Previous work has shown that reproductive queens from established polygyne colonies always possess genotype *Bb*, while reproductive queens heading monogyne colonies invariably possess genotype *BB* (Ross and Keller, 1998; Gotzek and Ross, 2007).

Chemical Extraction For piperidine analyses, queens were extracted individually in 80 μl of hexane (Fluka, Buchs, Switzerland) for 2 min., and then extracted in 80 μl dichloromethane (Riedel-deHaen, Seelze, Germany) for 11 min. Adult workers were extracted as groups of 200–500 individuals in 1 ml hexane for 2 min., and then in 1 ml dichloromethane for 11 min. Solvent from the extracts was evaporated under N_2, and the residue reconstituted with a known amount of hexane or dichloromethane.

We extracted individual queens and groups of workers from a second set of samples for hydrocarbon analysis. Queens were extracted in 80 μl hexane for 8 min. The solvent was evaporated under N_2, and the solute was re-dissolved in 2 μl hexane. Residual solvent on 14d-old queens was allowed to evaporate for 10 min., after which queens were stored at −80°C until determination of *Gp-9* genotype.

For hydrocarbon extraction of workers, subsets of 40 individuals were taken randomly from frozen colony samples and extracted in 100 μl hexane for 8 min. Solvent was removed from the extract by a N_2 stream, and the residue re-dissolved in 20 μl of hexane.

Chemical Analysis For piperidine analyses, extracts were first analyzed qualitatively on an Agilent (Santa Clara, CA, USA) 6890 gas chromatograph (GC), coupled with an Agilent 5975 mass selective (MS) detector operated in electron impact ionization mode. Peaks in extracts then were quantified by flame ionization detection (FID). GCs were operated in splitless injection mode, and were fitted with a DB-1MS column (30 m×0.25 mm×0.25 μm; Agilent). Column ovens were programmed from 60°–200°C at 40°C min^{-1}, after an initial delay of 2 min, then to 320°C at 5°C min^{-1}, and held at 320°C for 5 min. The injector was set at 260°C, the MS quad at 150°C, MS source at 230°C, and transfer line at 300°C. FID temperature was set at 320°C.

For hydrocarbon analysis, the MS was used exclusively, in order to identify some alkenes that differed with queen *Gp-9* genotype.

Chemical Identification Venom alkaloids and hydrocarbons were identified based on retention indices, mass spectra, and fragmentation patterns, with these data compared with previously published data (Brand et al., 1972, 1973; Nelson et al., 1980; Chen and Fadamiro, 2009; Dall'Aglio-Holvorcem et al., 2009). Double bond positions in unsaturated alkyl chains were determined by GC-MS fragmentation patterns of dimethyl disulfide adducts, as described in Liebig et al. (2009). See Table 1 for details of diagnostic ions.

Quantitative Analysis Peaks (Table 1) were aligned across samples, with only piperidines and unsaturated hydrocarbons used in the quantitative and statistical analyses. FID peak areas from both hexane and dichloromethane extracts were added, and the percentage of each peak out of total peak area calculated.

In the second set of samples, only hydrocarbon peaks were analyzed. We detected 49 compounds that were presumed to be hydrocarbons; this number was reduced to 26 for subsequent analysis. The peak area of all 49 presumptive hydrocarbons was summed and the relative proportion of each compound calculated. Then, the mean relative peak area of each compound was calculated for all queen and worker classes. For further analysis, we selected only compounds that had a mean of at least 1% of total peak area across all classes. The total amount of these 26 most abundant compounds was used as a reference for calculation of the relative proportions of each major unsaturated alkane. Peak areas of both data sets were calculated using MSD ChemStation D.02.00.275 data analysis software (Agilent Technologies).

Statistical Analysis For piperidines, Euclidean distances were used in non-metric multi-dimensional scaling (NMDS) analyses (Kruskal, 1964), using the program PRIMER 6.0. The first two dimensions of the model always resulted in stress values below 0.05, which is considered an excellent representation of data structure (Clarke and Warwick, 2001). The program STATISTICA 7.1 (StatSoft) was used to test for differences between monogyne and polygyne workers, and between monogyne and polygyne reproductive queens, in selected constituents

of the chemical profiles, using the *t*-test for independent samples (these data are normally distributed; Kolmogorov-Smirnov test, $P>0.2$).

Data for ratios of *cis*-C_{11}/(*cis*+*trans*)-C_{11} piperidines and peak areas of alkenes/alkadienes in queens were analyzed by means of resampling procedures coupled with binomial tests; resampling procedures were necessary because the use of multiple non-reproductive and reproductive queens from many of the polygyne source colonies meant that all replicates were not independent, violating a basic assumption of most standard statistical tests. Each class of queen was compared pairwise, with respect to ratios/peak areas, to each of the other classes, in the following way. For each iteration, a single replicate of each paired queen class was randomly selected and the difference in ratios/areas recorded; only paired replicates from different colonies were accepted. A third replicate of either of the two focal classes then was randomly selected from a different source colony than used for the first two replicates, and the difference between the two same-class replicate ratios/peak areas recorded. This procedure was repeated 1000 times (with replacement), and the paired between-class and within-class differences analyzed with binomial tests. The sample sizes, used for calculating the binomial probabilities following such resampling procedures, potentially range from the number of independent source colonies (most conservative) to the number of replicates (most liberal) used in the comparison; we chose to use the number of colonies in our calculations.

Results

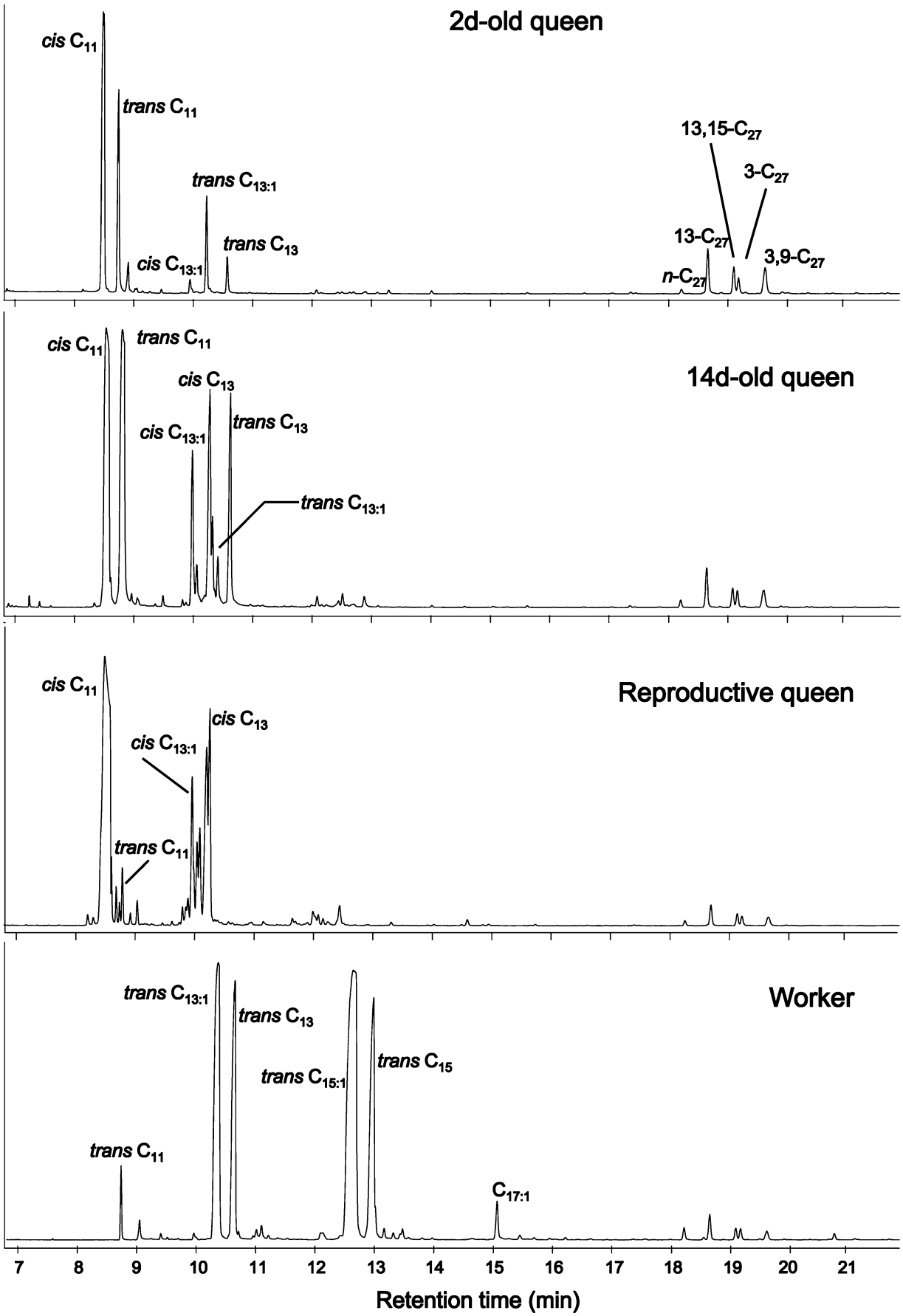
Differences in Venom Alkaloid Composition Among Females of Different Classes Qualitative differences were apparent in the venom alkaloid profiles of the different classes of *S. invicta* females (Fig. 1, Table 1). The 2d-old (sexually immature, non-reproductive) queens contained the lowest amounts of alkaloids relative to hydrocarbons, with the most abundant alkaloids being the two forms of C_{11} piperidine and, to a lesser extent, the *trans* configuration of C_{13} piperidines. The 14d-old (sexually mature, non-reproductive) queens also contained mostly C_{11} piperidines, but had greater proportions of both *cis* and *trans* C_{13} piperidines than younger queens. Reproductive queens lacked many of the *trans* piperidines. In contrast to queens, workers contained almost no C_{11} piperidines, but they had large amounts of longer-chain piperidines, especially C_{13} and C_{15} *trans* forms.

NMDS analysis confirmed these differences by showing clustering of piperidine profiles of the different classes (Fig. 2). Workers were separated completely from all types of queens, and workers originating from monogyne colonies were separated from those from polygyne colonies without overlap (2D stress value: 0.02). With the exception of a single individual (highlighted as an outlier in Fig. 3), reproductive queens also were completely separated from all types of non-reproductive queens. There was, however, no consistent difference between the reproductive *BB* (monogyne) queens and reproductive *Bb* (polygyne) queens. Similarly, there were no evident differences in piperidine profiles of the young non-reproductive queens of the different maturation stages and *Gp-9* genotypes.

Differences in Venom Alkaloid Composition Associated with Reproductive Status of Queens The proportion of *cis* piperidines (*cis*-C_{11}/totalC_{11}) was not different between 2-d- and 14d-old non-reproductive queens (Figs. 1, 3A). However, reproductive queens had higher proportions of *cis* piperidines than did either 2d- or 14d-old queens (Fig. 3A). There also was an effect of *Gp-9* genotype on proportion of *cis* piperidines in 14d-old queens, with *BB* queens having a higher proportion of *cis*-C_{11} than *Bb* queens (Fig. 3b). In this age class, the proportion of *cis* piperidines also was different between queens that had dealated and those that had not (Fig. 3b), in agreement with previous observations showing that *Gp-9 BB* queens dealate more readily than those with genotype *Bb* (Keller and Ross, 1999). Indeed, in the present study, there was also an association between *Gp-9* genotype and dealation, with 92.9% (26/28) of *BB* queens having dealated, while only 15.7% (8/51) of *Bb* queens dealated (*Fisher's exact test*, $P<0.001$).

Differences in Venom Alkaloid Composition Between Workers from Monogyne and Polygyne Colonies Adult workers from monogyne colonies had a higher proportion of saturated piperidines than workers from polygyne colonies (Fig. 4). This difference stemmed mainly from differences in saturated/total C_{13} piperidine ratios.

Differences in Cuticular Hydrocarbon Composition Associated with Gp-9 Genotype and Reproductive Status of Queens All castes possessed the typical hydrocarbons found in previous studies (e.g., Cabrera et al. 2004). However, we also found additional unsaturated compounds not previously reported (Fig. 5; Table 1). The following compounds met our criterion for detailed analysis: heptacosene, nonacosene, nonacosadiene, hentriacontene, and hentriacosadiene. As for the piperidine profiles, these compounds gave profiles of reproductive queens that were distinct from those of other classes of females. In particular, these compounds were completely lacking in workers (Table 1); worker hydrocarbon profiles differed from those of all queen classes (resampling procedures coupled with binomial tests, all $P<0.05$). Importantly, the total relative amounts of the


2d-old queen
cis C11
trans C11
trans C13:1
cis C13:1
trans C13
13,15-C27
3-C27
13-C27
3,9-C27
n-C27
14d-old queen
cis C11
trans C11
cis C13
cis C13:1
trans C13
trans C13:1
Reproductive queen
cis C11
cis C13:1
cis C13
trans C11
Worker
trans C13:1
trans C13
trans C15:1
trans C15
trans C11
C17:1
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Retention time (min)

◀ **Fig. 1** Representative total ion chromatograms of cuticular extracts of different classes of queens and of a worker of *Solenopsis invicta*. Venom alkaloids have retention times <17 min, whereas cuticular hydrocarbons have retention times >17 min. The 2d-old and 14d-old queens originated from polygyne colonies (*Gp-9* genotypes undetermined). The reproductive queen and worker profiles depicted are from individuals of the monogyne social form (*Gp-9* genotype *BB*). See Table 1 for definitions of the chemical acronyms

focal unsaturated hydrocarbons in queens varied according to *Gp-9* genotype and reproductive status, most dramatically between reproductive queens of the alternate (social forms) genotypes (Fig. 6). Reproductive *Bb* queens from polygyne colonies had a median of 17.7% of unsaturated hydrocarbons in their profile, while reproductive *BB* queens from monogyne colonies had a median of only 0.8% of unsaturated hydrocarbons, with one of the latter queens having no detectable amounts of unsaturated hydrocarbons.

While most (19/21) of 2d-old queens lacked any detectable amounts of unsaturated alkanes, the genotype-specific pattern of alkenes and alkadienes observed in reproductive queens had already begun to develop in queens over a 14-d period of maturation following eclosion (Fig. 6). Significantly, most 14d-old-*Bb* queens showed pronounced peaks of unsaturated hydrocarbons (median peak amount of 1.7%), while 14d-old-*BB* queens either lacked detectable amounts or had peak amounts of <0.1%. Thus, this difference in amount of unsaturated hydrocarbons, characteristic of reproductive queens of alternate genotypes, is paralleled by smaller differences in young, non-reproductive queens of alternate genotypes.

Discussion

This study shows that both venom alkaloid and hydrocarbon profiles in the fire ant *S. invicta* are associated with caste, queen reproductive status, queen *Gp-9* genotype, and colony social organization (Electronic supplementary material Fig. 7). Venom alkaloid composition changes dramatically, both qualitatively and quantitatively, as queens become reproductively active, and also differs between workers from monogyne and polygyne colonies. Cuticular hydrocarbon profiles also show pronounced differences among different classes of queens, with the qualitative and quantitative differences observed based mainly on the amount of alkenes and alkadienes. Differences in amounts of these unsaturated hydrocarbons are related not only to queen reproductive status, but also to *Gp-9* genotype of queens, with this latter difference already noticeable at 14 days post-eclosion, when queens are sexually mature but not yet reproducing.

Previous studies showed that the venom sac of reproductive fire ant queens contains mostly C_{11} alkaloids, which are barely present in the sac of workers (Brand et al., 1973). We found that the proportion of *cis* piperidines (the less abundant geometric configuration in workers), gradually increases in the venom of queens as they become fully reproductive. Moreover, in 14d-old (mature, but non-reproductive) queens, the proportion is higher in individuals of the *Gp-9 BB* genotype than in those of the *Bb* genotype. Similarly, alkenes/alkadienes are found in significant amounts only in the queen caste, and only polygyne reproductive queens contain copious amounts. It is important to note that the difference in amounts in 14d-old queens that we detected between *Bb* and *BB* queens is attributable to variation at the *Gp-9* gene (or variation at

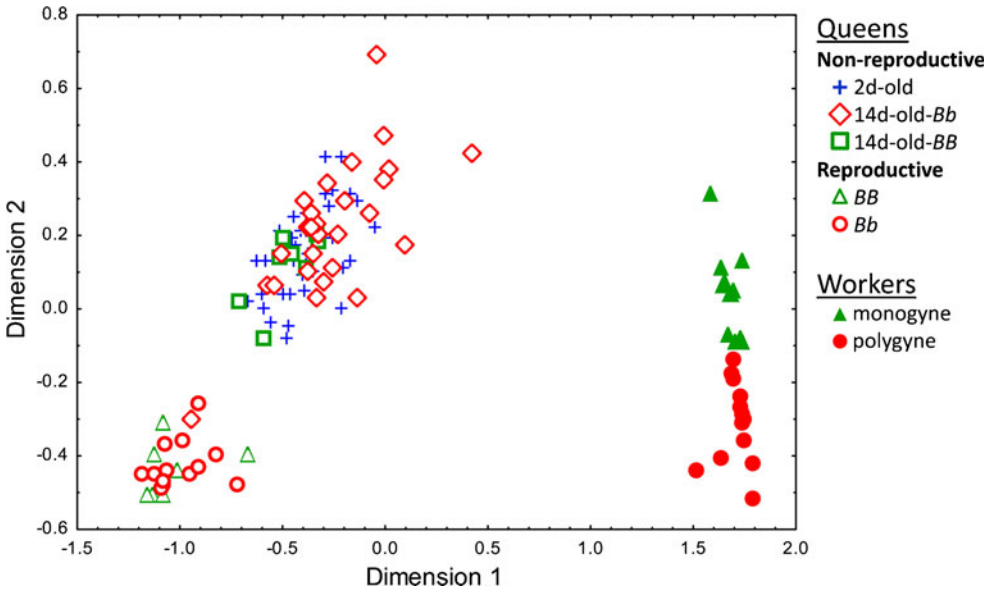


Fig. 2 Results of non-metric multi-dimensional scaling analyses of piperidine venom alkaloids of different classes of *Solenopsis invicta* females

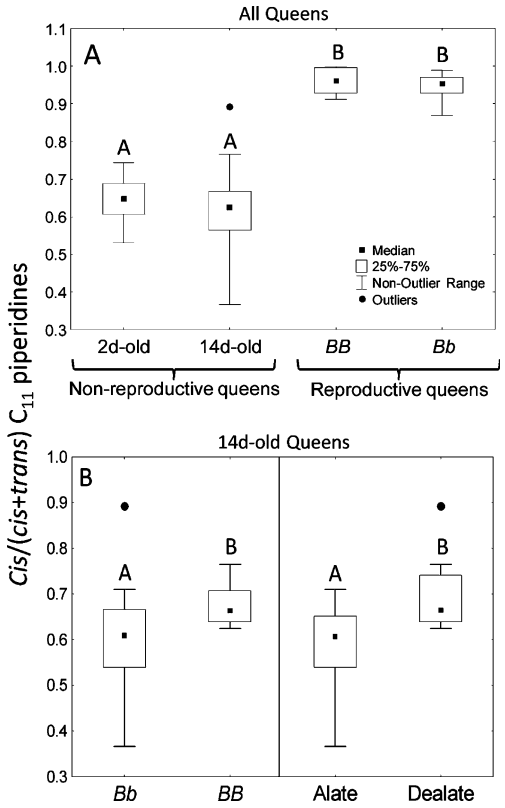


Fig. 3 Ratios of *cis*-C_{11}/(*cis*+*trans*)-C_{11} piperidines in different classes of *Solenopsis invicta* queens. Different letters within panels denote statistically significant differences. The outlier represents a dealate queen (included in the statistical analyses) evident in Fig. 2. **A** Patterns based on queen sexual maturity/reproductive status and on *Gp-9* genotype/social form of origin (resampling procedures coupled with binomial tests: 2d-old queens vs. *BB* or *Bb* reproductive queens, both $P<0.001$; 14d-old queens vs. *BB* or *Bb* reproductive queens, both $P<0.001$; 2d-old vs. 14d-old queens, $P=0.17$; *t*-test for independent samples: *BB* vs. *Bb* reproductive queens, $P=0.42$). **B** Patterns based on *Gp-9* genotype and dealation status of 14d-old queens (resampling procedures coupled with binomial tests: genotype *Bb* vs. *BB*, $P<0.001$; alates vs. dealates, $P<0.001$). *Cis*-C_{11}=*cis*-2-methyl-6-*n*-undecylpiperidine; *trans*-C_{11}=*trans*-2-methyl-6-*n*-undecylpiperidine

linked genes), rather than to effects of social environment, because all 14d-old queens in our study originated from polygyne source colonies. This initial difference, attributable to genotype, becomes more pronounced as queens age, accelerate oogenesis, and become fully competent reproductives.

That profiles of *cis* piperidines and unsaturated hydrocarbons differ between sexually mature *BB* and *Bb* queens suggests that these suites of chemicals constitute signals that workers use to recognize and discriminate between queens of the two genotypes. Importantly, such behavioral discrimination by workers only becomes apparent once queens become sexually mature (Ross and Keller, 1998; Gotzek and Ross, 2007), suggesting an association between ontogeny of these presumptive queen chemical signals and discrimination behavior directed toward them.

Previous studies showed that 14d-old-*BB* queens accumulate greater energy reserves, dealate more readily, and lay more eggs immediately upon dealation than 14d-old-*Bb* queens; i.e., sexually mature *BB* queens are at a more advanced state of reproductive development (Keller and Ross, 1993b; Ross and Keller, 1998; DeHeer, 2002). Thus, changes in alkaloid and hydrocarbon compositions are closely tied to physiological changes that accompany sexual maturation and the onset of reproduction that, in turn, are related to *Gp-9* genotype. Specifically, proportions of *cis*-C_{11} piperidines increase more rapidly during maturation in *BB* than *Bb* queens before attaining high proportions in reproductives of both genotypes, while amounts of alkenes/alkadienes increase more rapidly in maturing *Bb* than *BB* queens before reaching extremely high levels in *Bb* reproductives only. In line with this, 14d-old queens that dealated (i.e., those in a more advanced reproductive state, most of which lacked allele *b*) also had a higher proportion of *cis* piperidines than queens of the same age that had not dealated (most of which possessed allele *b*). Our results support tenets of the model of regulation of queen number proposed by Gotzek and Ross (2007), which invokes two distinct signaling components, one (piperidines) communicating actual or potential fertility status, and the other (alkenes/alkadienes) communicating *Gp-9* genotype. We note, however, that our data are not fully congruent with the model, which predicts that workers bearing the *b* allele should display the same chemical profile as queens bearing this allele; workers lacked the unsaturated hydrocarbons that were differentially abundant in queens of alternate *Gp-9* genotypes. There are several possible explanations for this incongruence, including the two classes of chemicals acting synergistically, rather than as distinct components, hydrocar-

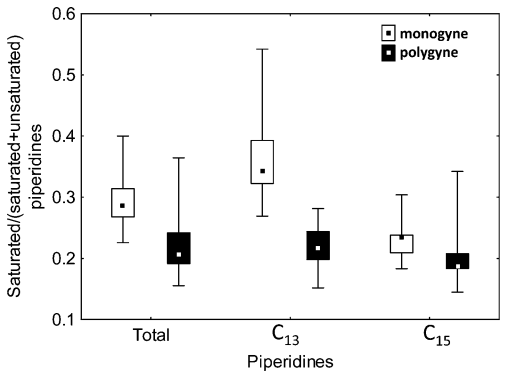


Fig. 4 Ratios of saturated to total piperidines in *Solenopsis invicta* workers from monogyne (*Gp-9* genotype *BB* only) and polygyne (mixed *Gp-9* genotypes) colonies. Comparisons of total piperidines, C_{13} piperidines, and C_{15} piperidines, respectively, are shown. Only total and C_{13} piperidines are different in ratios (*t*-test for independent samples: total, $t=3.46$, $P<0.003$; C_{13}, $t=6.05$, $P<0.001$; C_{15}, $t=1.72$, $P=0.098$)

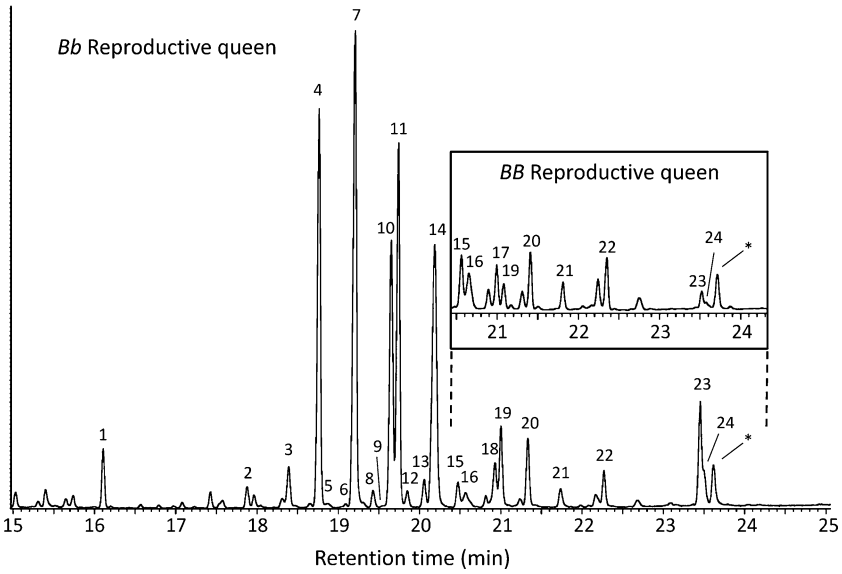


Fig. 5 Representative cuticular hydrocarbon profiles of *Solenopsis invicta* reproductive queens of *Gp-9 Bb* (polygyne queen) and *BB* (monogyne queen) genotypes. The highlighted partial profile of the *BB* queen is at the same scale as the profile of the *Bb* queen. *Numbers* correspond to compounds listed in Table 1. Any hydrocarbons eluting before 15 min are not shown. *Asterisk* indicates a contaminant

bon expression depending jointly on genotype and fertility status, or other classes of chemicals on the cuticle surface, including perhaps the GP-9 protein itself (unpublished data), being involved in signaling *Gp-9* genotype in *S. invicta*.

To date, fertility signaling has been assigned to cuticular and egg surface hydrocarbons in most social insects in which this effect has been investigated (Monnin, 2006; Le Conte and Hefetz, 2008; Peeters and Liebig, 2009; Liebig, 2010). In *S. invicta*, it appears that both hydrocarbons and piperidines may play roles in providing information relevant to regulation of colony queen number, as described above. Like hydrocarbons, piperidines are derived from acetate (Leclercq et al., 1996), raising the possibility that similar metabolic processes regulate the synthesis of hydrocarbon chains and piperidines.

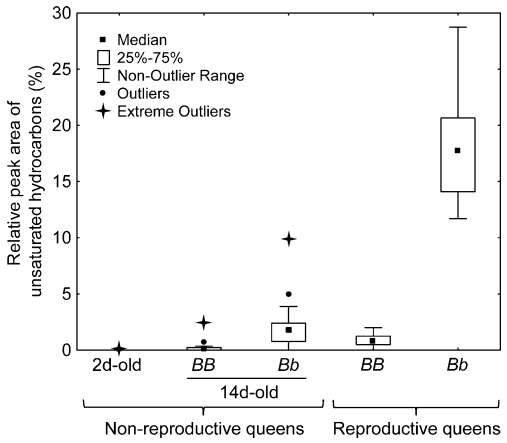


Fig. 6 Alkene and alkadiene relative amounts in different classes of *Solenopsis invicta* queens. All pairwise differences are significant (resampling procedures coupled with binomial tests: 2d-old queens vs. 14d-old-*BB* queens, and 14d-old-*Bb* queens vs. *BB* reproductive queens, both $P<0.05$; 14d-old-*Bb* queens vs. 2d-old and 14d-old-*BB* queens, both $P<0.01$; all other combinations, $P<0.001$)

Previously, piperidines were found not to be attractive in *S. invicta* (Vander Meer et al., 1980). The attractiveness of the queen poison gland secretion is attributed to two pyranones identified by Rocca et al. (1983a,b). Nevertheless, these pyranones do not delay the onset of reproduction in young queens, a primer effect that the poison sac was found to produce (Fletcher and Blum, 1981; Glancey et al., 1984; Vargo, 1998). Piperidines, with their large amounts dominating poison sac content, could be potential players in this phenomenon.

The ratio of saturated piperidines in adult worker cuticular extracts was higher in *S. invicta* workers from monogyne colonies than in workers from polygyne colonies (Fig. 4). This is in agreement with previous findings of differences in alkaloid composition between workers of the two social forms (Lai et al., 2008; Lin et al., 2010). Quantitative differences in piperidine composition have also been found between minor and major workers, as well as among workers of different ages (Deslippe and Guo, 2000), suggesting that piperidines also may be used for caste and possibly nestmate recognition.

Many ants use secretions from the metapleural gland as antiseptics for their nest and body surface. Fire ants have fatty acids with antimicrobial properties in the metapleural glands that may be used for that purpose (Cabrera et al., 2004), although venom alkaloids also may take part in defense against microbial pathogens. Workers of *S. invicta* display gaster-flagging behavior, whereby they spray the contents of the poison gland onto the nest and their body (Obin and Vander-Meer, 1985). Indeed, venom alkaloids dominate in solid phase microextraction (SPME) extracts of *Solenopsis xyloni*, even when the gaster cuticle is excluded

from extraction (unpublished data). As high molecular weight compounds, these alkaloids are nonvolatile (MacConnell et al., 1971) and potentially can remain on the cuticle for long periods. Considering the above, it is not surprising that piperidines also are found in abundance throughout the nest (Chen, 2007). The large quantities of venom alkaloids produced for a possible role in protecting ants from microbial pathogens may have facilitated their co-option to serve as fertility signals within colonies, especially if a queen's venom becomes distributed on her cuticle. Thus, the present study suggests that venom alkaloids of fire ants may have secondarily evolved roles in communication that typically are assigned to cuticular hydrocarbons in many other social insects (Monnin, 2006; Le Conte and Hefetz, 2008; Peeters and Liebig, 2009; Liebig, 2010). Despite the large amounts of alkaloids, hydrocarbons still may play a role in regulating social structure by signaling queen *Gp-9* genotype. Future manipulative behavioral studies should directly examine whether, and if so how, these and other compounds found on the queen cuticle, including GP-9 protein, function in maintaining the structure of fire ant societies.

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consequently, a young queen will take off for nuptial flight (Engels and Engels, 1984, 1988; Engels, 1987; Roubik, 1990; Sommeijer and De Bruijn, 2004).

Pheromones of virgin queens are believed to trigger DCA formation (Sommeijer and de Bruijn, 1995; Imperatriz-Fonseca et al., 1998; Veen Van and Sommeijer, 2000a), although definitive experimental proof has yet to demonstrate this. *Tetragonisca angustula* DCAs have been observed near nests (Sakagami, 1982; Engels and Engels, 1988; Veen Van and Sommeijer, 2000a; Galindo-López and Bernhard-Kraus, 2009), but rarely elsewhere in the field. Consequently, little is known about how mating occurs (Imperatriz-Fonseca et al., 1998; Veen Van and Sommeijer, 2000b). In order to learn more about *T. angustula* mating behavior, we established meliponaries with virgin queens in different habitats during the main male-production period (Velthuis et al., 2005). Curiously, after establishing these meliponaries, and while preparing new colonies to raise additional virgin queens, drones from a DCA located about 7 m away were attracted to a single physogastric (i.e., fertilized) queen previously removed from a queen-right colony (unpublished data). This led us to wonder whether queen volatiles were responsible for drone attraction.

Until now, there have been only two reports on drone attraction in stingless bees. The first was with *Scaptotrigona postica* (Engels et al., 1990), in which an artificial mixture of secondary alcohols (present in cephalic volatiles found in virgin queens) was shown to attract drones in an aggregation in the field. The second, with *S. mexicana* (Verdugo-Dardon et al., 2011), used both field and laboratory experiments, and showed that drones differentiated between virgin and physogastric queen volatiles originating from the head. Virgin queen cephalic extract elicited strong drone antennal and behavioral responses.

In this study, we observed *T. angustula* drone assembling behavior, and examined drone behavioral and electrophysiological responses to natural and synthetic volatiles of virgin and physogastric queens. We also recorded the relative abundances of volatiles in regard to queen reproductive status, and located the source of volatiles in the queen body.

Methods and Materials

Study Site and Meliponaries Experiments were carried out in the Soconusco region, Chiapas, Mexico, from Dec. 2009 to March 2011. Four *T. angustula* meliponaries were established in two sites, eight km apart. Site 1) agroforestry cacao plantation, with two meliponaries, Izapa (14°54′N and 92°11′W) and Medio Monte (14°53′ N and 92°11′ W), with seven colonies in each site, 2.8 km apart; Site 2) Urban area with two meliponaries, Downtown (14° 54″ N and 92° 16″ W) and ECOSUR (14°53′ N and 92°17′ W), with five and seven colonies, respectively, 2 km apart. Each colony contained at least one recently emerged virgin queen. Meliponaries were established by Dec 2009, with the exception of ECOSUR, which was established in Dec 2010 at the El Colegio de la Frontera Sur (ECOSUR) facility. Most of the work reported here used colonies at the urban ECOSUR site.

Bee Colonies and Recording All colonies in the meliponaries were removed from feral nests and established in wooden boxes (30×20×10 cm) from which the queen was removed. The queenless colonies contained 500–1,000 workers, few drones, several combs with emerging broods, at least one mature queen cell, and sufficient food stores. The meliponaries were observed daily for drone congregations. After the first DCA was formed, we checked every 2 h between 08:00 and 18:00 h, and recorded the number of drones. Ten bait hives in each site, located to attract swarms of *T. angustula*, were hung 1–15 m from the occupied hive. These bait hives were checked daily to determine whether new colonies had settled in them (Engels and Imperatriz-Fonseca, 1990).

Collection of Volatiles and Sample Preparation Individual virgin (1–5 d-old; $N=6$) and physogastric ($N=17$) queens (both types taken directly from the nest and assayed immediately), as well as individual heads, thoraces, and abdomens from queens ($N=3$), were placed in 20 ml Erlenmeyer flasks with an aluminum foil lid. Queen bees were left in flasks for 30 min before volatile collection. Volatiles were collected from the flasks for 30 min. at ambient temperature using a Solid Phase Micro-Extraction method (SPME) fiber (65 mm polydimethylsiloxane divinylbenzene; Supelco, Bellefonte, PA, USA). For extracts, an individual intact queen (virgin or physogastric), or dissected body parts (head, abdomen, and thorax), was crushed in 400 µl of dichloromethane. Extracts were concentrated to 100 µ1 (queens and abdomen) or 50 µl (head and thorax) per queen equivalent by a nitrogen stream, and stored at −20°C until used for analysis or bioassays.

Electrophysiology Gas chromatography-electroantennographic detection (GC-EAD) analyses were performed using SPME volatile collections of living queens and queen body parts, as well as 2 µl of synthetic isopropyl hexanoate (IPH; 2 mg/ml). Antennae from drones caught from a DCA, or workers (nurse and forager) from established colonies, were carefully removed, and the base was inserted into a reference glass capillary electrode, previously filled with saline solution (Malo et al., 2004). The distal end of an antenna was inserted into the tip of the glass recording capillary electrode. The signal generated by

an antenna was passed through a high-impedance amplifier (Syntech NL 1200, Hilversum, The Netherlands) and displayed on a monitor using Syntech software for processing GC-EAD signals. One drone or worker antenna constituted one sample (N=10).

Chemical Analyses To identify EAD-active compounds, volatiles and extracts were analyzed on a Varian Star model 3400 CX GC (Palo Alto, CA, USA). A DB-5 column (30 m×0.25 mm ID) was temperature programmed from 50°C (held for 2 min) to 250°C at 15°C min^{-1}. The injection port temperature was held at 200°C. The GC was coupled to a Varian Saturn 4D mass spectrometer and integrated data system. Ionization was carried out by electron impact at 70 eV, 230°C. Mass spectral identifications of the compounds were confirmed by comparison of retention times and mass spectra with those of synthetic standards. The relative amount of each compound was calculated from peak area, while the relative percentage of a component was calculated relative to the sum of all peak areas.

Preparation of Esters Esters were prepared by heating the corresponding alcohol (10 μl), carboxylic acid (5 μl), and acetic acid (2 μl) together with a drop of concentrated sulfuric acid in a Keele microreactor (Attygalle and Morgan, 1986) for 12 h at 100°C. The reaction was neutralized with $NaHCO_3$, and esters extracted with hexane (200 μl). IPH was synthesized from isopropyl alcohol (Merck, Mexico, S.A., chemical purity 99.7%) and hexanoic acid (Sigma-Aldrich, Toluca, Mexico, chemical purity 99%); butyl hexanoate (BH) from butanol (Productos Quimicos, Monterrey, Mexico, chemical purity 99.4%) and hexanoic acid; hexyl hexanoate (HH) from hexanol (Sigma-Aldrich, Toluca, Mexico, chemical purity 98%) and hexanoic acid.

Behavioral Bioassays To test the attractiveness of samples from both virgin and physogastric queens, bioassays were conducted in the ECOSUR bee yard. Live physogastric queens (N=5), in a wire cage on a 1.5-m-high tripod, and a 5 mm diam. cotton ball in another wire cage (used as positive and negative controls, respectively) were exposed three times, each 1 min., daily around noon, with breaks of 30 min between exposures. These tests were conducted over 5 d at a distance of 1, 3, 5, 7, 10, 15, and 20 m from DCA 3 (containing ~2,000 drones, estimated by counting the number of males that visited the cages). Virgin queens (N=3), 3–7 d-old were exposed on 3 different days, as described above, to DCAs 3 and 6 (each with ~2,000 males).

A short distance, two-choice bioassay was carried out to test the attractiveness of parts of the queen body (head, thorax, and abdomen; N=3), as well as queen extract, and IPH alone or combined with BH and HH. A queen, a part of the queen body, a cotton ball impregnated with one queen equivalent, IPH (0.4 mg) alone, or a blend of IPH (0.4 mg), BH (0.2 mg) and HH (0.2 mg), was presented on a stand (0.5 m from DCA 7 with ~150 drones), along with a blank cotton ball (control), around noon for 5 min. A drone's response was classified as "attract" when it flew upwind toward and within 5 cm of a sample; "touch" when it had brief contact with a cotton ball or a wire cage; and "mount" when it attempted to copulate. To avoid habituation, cotton balls and wire cages were changed after each test. Each queen was used only once per day.

Statistical Analysis The significance of drone attractiveness to both queen statuses at different distances, as well as the drone responses to the queen body parts and synthetic blend or alone, were calculated using the Wilcoxon T test with continuity correction. This method is contained in the software *R* Development Core Team (2010). A significance level of 5% was used.

Results

Drone Congregation Areas A total of eight *T. angustula* DCAs were observed during the experiment. Two established in the "Downtown" site and six in the "ECOSUR" site, both in urban areas. Three DCAs had more than 2,000 males during the days of highest assemblage. DCAs remained in place for up to 35 d (Table 1). The DCAs formed on different substrates, such as a clay tile, a wooden planter with some bees on overhanging vegetation, or a hive, but always close to (~0.5 m) or beside colonies or bait hives with a recently established swarm containing a virgin queen. The mean number of drones arriving the first day was 190 (±147). Males arrived daily at the congregation site from 08:00 to 18:00 h, with peak arrival time between 11:00 and 14:00 h. No DCA was observed at any of the two (Izapa and Medio Monte) meliponaries in the agroforest location.

Electrophysiology Queen volatiles, collected by SPME from either virgin or physogastric queens, elicited strong signals from *T. angustula* drone antennae (Fig. 1a and b, respectively), but not from females (nurses and foraging workers, data not shown). At least one EAD-active compound was apparent (retention time, 6.7 min) from both types of queens. This compound was present in the abdomen of queens. Antennal responses to extracts of intact queen or dissected abdomens elicited a weaker signal than the SPME collections. Although traces of the active

Table 1 Establishment of Drone Congregation Areas (DCA) of *Tetragonisca angustula* at urban areas in the Soconusco Region, Chiapas, Mexico

DCA	Arrival date	Total drones first day	# Drones largest assembly	Days to largest assembly	*Substrate used for assembly	DCA duration (days)
1	12/12/2009[1]	200	500	3	†Hive	5
2	02/20/2010[1]	80	250	3	Wood planter	4
3	01/23/2011[2]	150	2,400	3	†Hive	19
4	01/31/2011[2]	80	500	5	†Hive	5
5	02/06/2011[2]	150	800	2	‡Bait hive	4
6	02/11/2011[2]	500	2,200	24	‡Bait hive	34
7	02/15/2011[2]	300	2,000	13	‡Clay tile	35
8	03/19/2011[2]	60	60	1	†Hive	3

Key: †Colonies containing virgin queens; ‡ Bait hive with new colony with virgin queen; [1] DCAs in downtown site; [2] DCAs in ECOSUR site; *Substrate with same letter mean same location

compound were found in the thorax and head, no EAD responses to volatiles collected from these parts were obtained. Synthetic IPH elicited a discernible EAD response from drone (Fig. 1c), but not female, antennae (data not shown).

Chemical Analyses Typical gas chromatograms of SPME volatile collections from live virgin and physogastric *T. angustula* queens are presented in Fig. 1a and b, respectively. Three major peaks were identified: IPH [retention time: 6.7 min., major *m/z* (relative abundance,%): 43(100), 56(47), 84(41), 99(44), 117(50)]; BH [retention time: 8.3 min., *m/z* 43(60), 56(100), 71(29), 99(78), 117(57)], and HH [retention time: 10.3 min., *m/z* 43(100), 60(40), 71 (20), 99(46), 117(22)]. Chemical analyses of the two types of queens were similar qualitatively, but differed quantitatively, with virgin queens characterized by an abundance of IPH, a small amount of BH, and a trace of HH, while physogastric queens had relatively large amounts of IPH and HH, and a small amount of BH (Table 2). Interestingly, young, recently mated queens (2–4 wk-old) had relative amounts of HH in between the other two types (Table 2). The composition in the abdomen of virgin queens was similar to that for live queens, whereas the thorax and head had only traces of IPH and HH, as well as several compounds not found in the abdomen (Table 2).

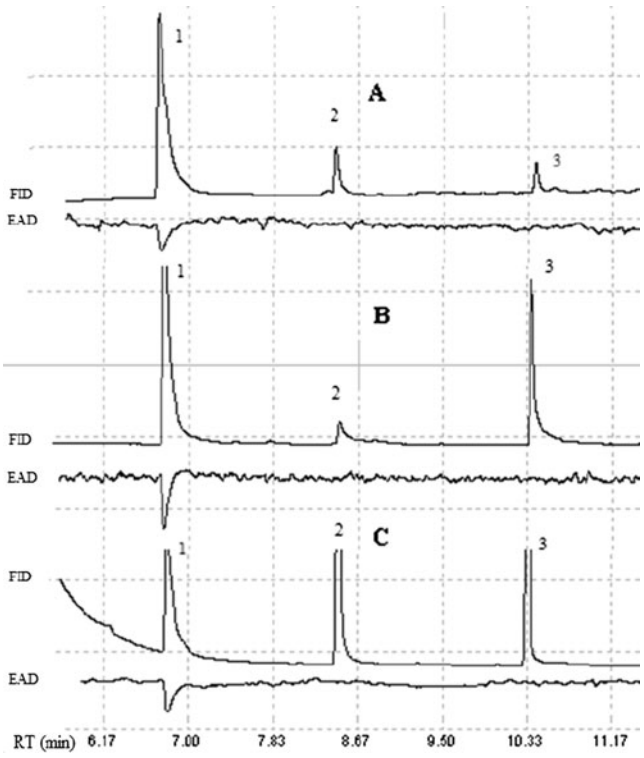


Fig. 1 Coupled gas chromatographic (with flame ionization detection; FID) and electroantennographic (EAD) responses of *Tetragonisca angustula* males to virgin (**a**) and physogastric queen (**b**) volatiles, or to a blend of synthetic compounds (**c**). Peaks: 1=isopropyl hexanoate, 2=butyl hexanoate, 3=hexyl hexanoate

Table 2 Relative abundance* of compounds in intact virgin and physogastric *Tetragonisca angustula* queens (±SD) and their body parts

Queen status	Peak 1** (IPH)	Peak 2 (BH)	Peak 3 (HH)
Virgin (N=6)	44.2 (±16.4)	7.8 (±3.2)	6.6 (±6.5)
Young phys (N=4)[1]	37.4 (±16)	5.3 (±2.7)	12.9 (±4.4)
Physogastric (N=13)	38.9 (±9.9)	8.1 (±2.4)	26.2 (±6.4)
Abdomen (N=3)[2]	22.4 (±13.9)	3.53 (±4)	8.6 (±4.7)
Thorax (N=3)[2]	8.3 (±8.1)†	1.7 (±1)†	1 (±0.9)†
Head (N=3)[2]	Traces	Not found	Not found

Keys: *According to area peak size; **Electroantennogram-active compound; [1] Young physogastric queen 4–6 wk-old; [2] from virgin queens; †possible contamination from abdomen. *IPH* isopropyl hexanoate; *BH* butyl hexanoate; *HH* hexyl hexanoate

Drone Behavioral Bioassay with Living Queens and Synthetic Compounds Drones were attracted to live virgin and physogastric queens over various distances. Both virgin and physogastric queens were detected by drones when slowly brought closer to the DCA (~2,000 males), with attraction increasing the closer the queens were exposed to the DCA (Fig. 2). At 20 m from the DCA, no males were attracted to queens.

Of the three body parts tested in the bioassay, the greatest number of responses occurred to the abdomen of a virgin queen, with fewer males responding to the thorax and none to the head (Table 3). The attract, touch, and mount responses to the abdomen and thorax were greater than the corresponding responses to the control. Only a few seconds after exposing the cage containing a queen's abdomen, drones flew upwind and touched or attempted to mount it, with fairly consistent responses recorded over each of the 5 min of the bioassay (Table 3). One equivalent of virgin queen extract attracted a significant number of males (relative to the control), of which only two (not different from that to the control) touched the cotton ball containing the extract. Relatively high numbers of drones were attracted to synthetic IPH, but few (three, not different from the control) of these actually touched the cotton ball. Similar responses were obtained to the mixture of IPH, BH and HH. The blank (control) did not attract any drones (Table 3).

Discussion

Tetragonisca angustula male aggregations are common, usually establishing during the reproductive stage of colonies. However, it is rare to find wild *T. angustula* DCAs, and consequently, not much is known about how mating occurs (Imperatriz-Fonseca et al., 1998; Veen Van and Sommeijer, 2000b). In our study, we found two *T. angustula* DCAs established in the "Downtown" meliponary and six DCAs in the "ECOSUR" meliponary, both urban areas. All DCAs were established during the main drone production period, as observed in other stingless bee species (Velthuis et al., 2005). The DCAs were found close to nests containing a virgin queen and located on a variety of substrates, as known for several stingless bee species (Sakagami, 1982; Engels and Engels, 1988; Veen Van and Sommeijer, 2000a; Sommeijer and De Bruijn, 2004; Galindo-López and Bernhard-Kraus, 2009). These results suggest that the presence of at least one virgin queen is a primary element that triggers the onset of DCA formation (Sommeijer and de Bruijn, 1995; Imperatriz-Fonseca et al., 1998; Veen Van and Sommeijer, 2000a). However, it seems that it is not the only factor, as males still aggregated at the same site even after the queen was fertilized. We speculate that *T. angustula* drones may leave "markings" that are used in the orientation of other drones toward a given site, as observed in *S. mexicana*. In this species, once a site is selected, the aggregation remains in the same place regardless of the queen's status (virgin, physogastric, or even queenless) (Galindo-López and Bernhard-Kraus, 2009).

We observed no DCAs formed in the agroforest meliponaries, suggesting that the density of feral nests, as well as spatial distribution, plays a crucial role in the formation of aggregations. *Tetragonisca angustula* uses a broad diversity of substrates for nesting, and is thus well suited to urban environments (Noll et al., 1993; Sousa et al., 2002). In our study areas, *T. angustula* was three times more abundant in urban areas, and was distributed relatively homogenously, than in the agroforest areas, where it has a more random spatial distribution (Fierro et al., in press).

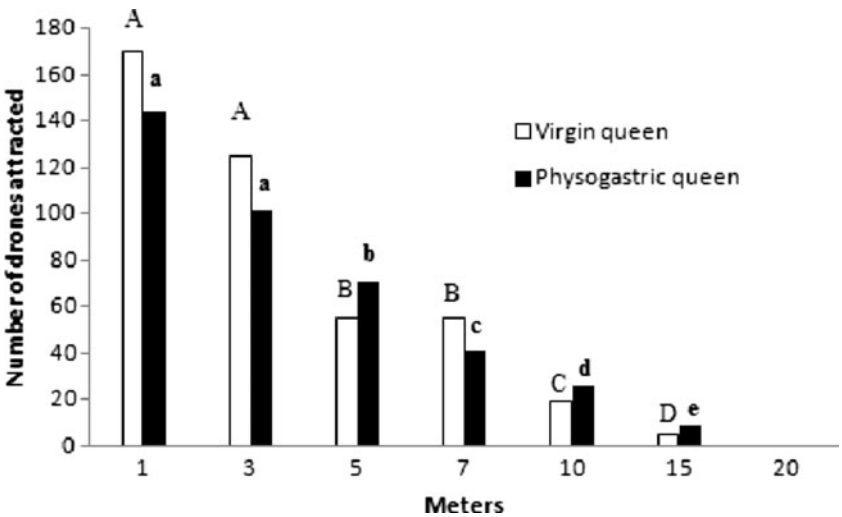


Fig. 2 Numbers of drones, from large congregations of ~2,000 males, attracted to virgin (N=9) (*light bars*), and physogastric (N=15) (*dark bars*) *Tetragonisca angustula* queens. Queens were exposed for 1 min at different distances from the drone congregations. *Same letter* of the same case, among drone responses at different distances, indicate no statistical difference (P>0.05; Wilcoxon T test with continuity correction)

Table 3 *Tetragonisca angustula* drone responses to virgin queen body parts, whole queen extract, or synthetic chemicals

Time	Abdomen			Thorax			Head	Queen extract			Synthetic (IPH)			Synthetic blend			Cotton ball
(min)	Attract*	Touch*	Mount*	Attract*	Touch*	Mount*		Attract*	Touch	Mount	Attract*	Touch	Mount	Attract*	Touch	Mount	Control**
1	25	8	17	8	3	5	0	3	0	0	7	0	0	4	0	0	0
2	33	12	21	5	2	3	0	2	1	0	5	1	0	5	0	0	0
3	28	5	13	6	2	4	0	4	0	0	13	0	0	3	0	0	0
4	27	9	18	8	3	5	0	4	1	0	8	2	0	6	1	0	0
5	31	12	19	4	2	2	0	5	0	0	3	0	0	1	0	0	0

IPH=isopropyl hexanoate (IPH); synthetic blend = IPH + butyl hexanoate + hexyl hexanoate(HH)

* Response different ($P<0.05$) from that to the control

** No drones responded (attract, touch, or mount) to the control

With the exception of DCA 8, all our drone aggregations were formed by greater than several hundred males. Such large numbers of drones should ensure that queens mate with an almost panmictic sample and therefore avoid inbreeding (Baudry et al., 1998). The stay of drones at a DCA generally lasts only a few days, with males disappearing after the queen has been fertilized (Veen Van and Sommeijer, 2000a). However, three of the six DCAs we found (Table 1), and remained in the same place for 19–35 d. This behavior is similar in DCAs observed in meliponaries of *S. mexicana*, in which DCAs remained in the same place for 24 d (Galindo-López and Bernhard-Kraus, 2009). This long stay may occur because of the intensive handling of bee colonies within a meliponary (preparing new colonies to raise new virgin queens).

The range of attraction (1–20 m) of drones we observed in our bioassay is consistent with that of other stingless bee species (Engels and Engels, 1988; Sommeijer and De Bruijn, 2004) and congruent with the successful formation of DCAs seen in urban areas. The efficiency of queen-male chemical communication is amplified by male-male communication, and the uniform spatial distribution of nests in urban areas favors DCA formation (Fierro et al., in press). Curiously, when we were preparing colonies to raise new virgin queens, drones from a DCA (>2,000 males) were attracted to a single physogastric queen removed from a queenright colony and placed in a wire cage (unpublished data). This attraction was similar to that observed to virgin queens, and may indicate that polyandry occurs in this species, as reported for several other species of solitary and social Hymenoptera (Page and Metcalf, 1982; Page, 1986). Queens of highly eusocial bees mate only during a short period at the beginning of their reproductive lives. For example, queens of *Apis* species copulate with an average of 23 drones, during one or more mating flights, and then start oviposition (Adams et al., 1977). In contrast, stingless bee queens have long been thought to mate only once, e.g., *M. quadrifasciata* (Kerr, 1969; Silva et al., 1972; Peters et al., 1999). However, mating in stingless bees has been studied in only a small number of species (Lopes et al., 2003), and evidence of attraction to physogastric queen volatiles (Engels and Engels, 1988; Campos and Melo, 1990) may indicate that queens of some species do mate more than once.

Both virgin and physogastric queens released the same three esters, but in different relative amounts. Engels et al. (1990) and Verdugo-Dardon et al. (2011) found a similar result in cephalic chemistry between virgin and physogastric queens in both *S. postica* and *S. mexicana*. In our study, the most obvious difference between virgin and physogastric queens was the relative amount of HH. This compound had elevated levels in recently mated queens. In *S. mexicana* queens, HH became the most abundant com-

pound once a queen was fertilized and began egg laying (Grajales-Conesa et al., 2007). Thus HH levels may be a marker for a queen's reproductive state, and perhaps may be involved in regulating colony organization and worker behavior. Further work is needed to test this.

While HH has previously been found in *S. mexicana*, the other two compounds have not been found in other stingless bees. Butyl hexanoate is used as a sex pheromone in *Megalotomus quinquespinosus* (Heroptera, Alydidae, Alydinae), and as a kairomone in *Cydia pomonella* and *Grapholita molesta* (Lepidoptera, Tortricidae), (El-Sayed, 2011). Isopropyl hexanoate has been found in extracts of sexually mature *Drosophila putrida* (Diptera, Drosophilidae) males, while in *D. recens* it is a sex pheromone (Jaenike et al., 1992). Isopropyl hexanoate is widely distributed in flower essences where its evolutionary function is to attract pollinators (Dobson and Bergström, 2000).

Although *T. angustula* males showed strong attraction to IPH, either alone or in combination with BH and HH, males exhibited little touching. Since isolated abdomens both attracted and elicited males to mount, this suggests that there may be other close-range olfactory stimuli involved in eliciting more complete behavior. Similar results have been found in Apidae. For example, with the bumblebee, *Bombus terrestris*, a blend of synthetic virgin queen pheromone elicited male attraction but did not provoke "mounting" (Krieger et al., 2006). In honey bee queen pheromone studies, 9-ODA (a queen sex pheromone component) attracted males (Butler and Fairey, 1964), but this compound alone was less attractive than an intact queen (Slessor et al., 1988). In *S. postica*, a mixture of four synthetic secondary alcohols, placed on a dummy, elicited only occasional "touching" when presented to a drone congregation (Engels et al., 1990). The presence of additional behaviorally active compounds produced by queens should be investigated.

We have shown that volatiles from both virgin and physogastric *T. angustula* queens attract and influence the behavior of drones. This attraction is caused, at least in part, by IPH released from the abdomen of a queen. However, further research is needed to elucidate any biological function of the presence of this compound in *T. angustula* physogastric queens, as well as its possible effect as a queen pheromone in the reproduction of the highly eusocial Meliponini.

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mediators of two of the hallmarks of sociality: the queen-worker reproductive division of labor, and worker task specialization (Wilson, 1971). Furthermore, sociality and the accompanying pheromone-communication systems have evolved independently multiple times in the order Hymenoptera (bees, ants, and wasps), allowing comparative approaches for understanding the evolution of these systems (Wilson, 1971; Ross and Matthews, 1991; Brady et al., 2006; Hines et al., 2007). It is likely that these complex communication systems evolved from systems that regulate more basal pheromone-mediated behaviors, such as mating and defense, which are found even in solitary lineages of wasps (Blum, 1969). Thus, chemical cues associated with physiological status, particularly reproductive state, may have been co-opted as signals that regulate social status (reviewed in Wyatt, 2003).

In the last 10–15 years, a great deal of progress has been made in identifying pheromones and characterizing pheromone responses, particularly at the molecular level. Here, we build on previous reviews of pheromones in ants and honey bees (Monnin, 2006; Le Conte and Hefetz, 2008; Heinze and d'Ettorre, 2009; Holman, 2010; van Zweden, 2010), and provide an overview of recent advances in our understanding of pheromone systems that regulate queen-worker interactions in multiple social insect species, including honey bees, bumble bees, wasps, ants and termites. We further discuss how this information impacts our understanding of the ultimate mechanisms that underlie pheromone communication. Finally, we discuss how recent advances in genomics have contributed to our understanding of the mechanisms that regulate pheromone communication, and how these tools may be used in the future to elucidate further the evolution of these sophisticated chemical communication systems.

The Role of Pheromones in Regulating Reproductive Division of Labor The reproductive division of labor found in social insects leads to conflicts between individuals over reproductive status (reviewed in Ratnieks et al., 2005). Kin selection theory (Hamilton, 1964) proposes that these conflicts are resolved in part because sterile workers increase their inclusive fitness by rearing related, reproductive nestmates to offset the loss of direct fitness. However, conflict can still exist, especially in the case of male production. In smaller colonies, where individuals can interact directly with each other, physical dominance interactions may have evolved as a means to resolve these conflicts through social hierarchies, and, in some cases, these appear to have been reinforced by pheromones (West-Eberhard, 1967; Wilson, 1971; Keller and Nonacs, 1993). In many primitively eusocial wasps and bees, there are no morphological differences between castes, and reproductive dominance tends to be established by one or a few individuals through behavioral dominance or food restriction, which prevent full reproductive development (West-Eberhard, 1967). In *Polistes* wasps and permanently queenless ants, aggression by the dominant reproductive individual limits both mating opportunities and reproduction of subordinates (Reeve, 1991; Roeseler, 1991; Peeters, 1993; Monnin and Peeters, 1998). However, after reproductive dominance is established, a reduction in aggression levels often is observed, which may be mediated by chemical cues, at least in *Polistes dominulus* (Sledge and Boscaro 2001) and *Bombus terrestris* (Sramkova et al., 2008; Röseler and Van Honk 1990; Amsalem and Hefetz, 2010). In bumble bees, *Bombus terrestris*, direct contact with the queen is necessary for the inhibition of worker reproduction. However, these interactions do not appear to be aggressive in nature (Alaux et al., 2004; Lopez-Vaamonde et al., 2007), suggesting that a contact pheromone that inhibits worker reproduction may be present. In the sympatric, primitively eusocial paper wasp species, *Ropalidia marginata* and *Ropalidia cyathiformis*, it appears that *R. cyathiformis* queens rely primarily on physical aggression to establish reproductive dominance, while *R. marginata* queens appear to use chemicals produced in the Dufour's gland to mediate reproductive dominance, and are not physically aggressive (Kardile and Gadagkar, 2002; Bhadra et al., 2010).

In larger, more derived societies, such as honey bees, vespine wasps, and several species of ants, queen pheromones are used in combination with other types of pheromones (Pettis et al., 1997) and worker policing (Ratnieks and Visscher, 1989) to maintain the reproductive division of labor. As a result, few aggressive interactions are observed among queens and workers (Fletcher and Ross, 1985; although see Smith et al., 2009 for reproductive inhibition by aggression in a highly-eusocial ant). However, detailed studies of these pheromones are rare, and have focused primarily on two species: the fire ant, *Solenopsis invicta* (reviewed in Vargo, 1998), and the honey bee, *Apis mellifera* (reviewed in Le Conte and Hefetz, 2008). Recently, queen pheromones also have been identified in a termite (Matsuura et al., 2010) and in the ant *Lasius niger* (Holman et al., 2010a, b).

Queen Pheromones Cuticular hydrocarbons (CHCs) appear to be associated with reproductive status in primitive and advanced social species and, in some cases, have been shown to function as pheromones. CHCs are a mixture of lipids that cover the insect body to prevent water loss and are modified by genotype, physiology, and environmental factors (reviewed in Howard and Blomquist, 2005). In solitary insects, CHCs may play a role in species recognition and identification of mating partners (Wyatt, 2003). CHCs are also critical for nestmate recognition in many social insect species; because CHCs are sensitive to physiological state, it is possible that their use as individual cues may have evolved into queen recognition cues and/or

fecundity signals (for further discussion, see Hölldobler and Michener, 1980; Hölldobler 1984).

In some halictid bees (*Lasioglossum malachurum*, Ayasse et al., 1993; 1999), paper wasps (*Polistes dominulus*, Sledge and Boscaro 2001), and queenless and ponerine ants (*Dinoponera quadriceps,* Monnin and Peeters, 1998; Peeters et al., 1999; *Diacamma ceylonense*, Cuvillier-Hot et al., 2001; *Harpegnathos saltator*, Liebig et al., 2000; *Gnamtogens menadensis* and *Diacamma* sp., Gobin et al., 1999, Kikuta and Tsuji, 1999), differences in CHC profiles among individuals within a colony correlate with differences in ovarian activation levels. Furthermore, differences in CHCs are associated with reproduction in lower termites. Interestingly, CHCs associated with fertility are similar in lower termites and Hymenopteran species studied, suggesting that these mechanisms may represent convergent evolution in chemical communication (Weil et al., 2009).

In only a handful of cases have CHCs been shown to act as functional pheromones that alter behavior and/or physiology. In the ponerine ant, *Pachycondyla inversa*, both fertile queens and dominant workers (in queenless colonies) produce 3,11-dimethylheptacosane. This compound is detected by antennae of workers, and is associated with individual differences in reproductive status, indicating that it is likely a signal of fertility (D'Ettore et al., 2004) although a behavioral bioassay is necessary to demonstrate true pheromonal activity. In *Lasius niger*, the common garden ant, a single CHC (3-methylentriacontane) inhibits worker ovary activation, decreases worker aggression, and elicits a strong response in worker antennae (Holman et al., 2010b). This same compound is present on queen-laid eggs.

Finally, CHC cues also may be used to discriminate different degrees of reproductive output. For example, in the polygynous colonies of *Formica fusca*, queens of different reproductive status (majority of production, minority of production, no production) exhibited differences in CHC profiles and associated worker responses (Hannonen et al., 2002). Queens with the greatest fecundity received the greatest amount of attention from all workers regardless of kinship.

Queen pheromones also may be composed of more chemically complex blends that can arise from multiple sources, including dedicated glands for pheromone synthesis. In the bumble bee species, *Bombus hypnorum*, queens and workers show significant differences in the amount and proportion of volatiles collected from multiple glandular and cuticular sources, and these differences are associated primarily with differences in ovarian activation levels and caste (Ayasse et al., 1995). Similarly, in *Bombus terrestris,* egg-laying queens have a unique chemical bouquet including certain queen-specific compounds (Sramkova et al., 2008). Honey bee queen pheromone is a blend of chemicals produced by multiple glands, including the Dufour's gland, the tergite glands, and the mandibular glands (Slessor et al., 2005; Le Conte and Hefetz, 2008). Five components, (*E*)-9-keto-2-decenoic acid (9-ODA), (*R*,*E*)-(−)- and (*S*,*E*)-(+)-9-hydroxy-2-decenoic acid (9-HDA), methyl *p*-hydroxybenzoate (HOB), and 4-hydroxy-3-methyoxyphenylethanol (HVA), of this pheromone have been identified (Slessor et al., 1988) in the mandibular glands of mated queens (queen mandibular pheromone or QMP), and together they elicit many of the same responses as a live queen, albeit with less potency.

Honey bee queen pheromone can elicit both primer and releaser responses. Primer pheromones elicit behavioral and/or physiological effects that occur over longer timescales, while releaser pheromones elicit immediate behavioral responses. As a primer pheromone, honey bee queen pheromone modulates worker ovarian activation (Hoover et al., 2003), and inhibits rearing of new queens by workers (Melathopoulos et al., 1996; Pettis et al., 1999). It also delays the transition from nursing to foraging (Pankiw et al., 1998), inhibits juvenile hormone synthesis in workers (Pankiw et al., 1998), influences comb building within the colony (Ledoux et al., 2001), and has effects on worker brain gene expression (Grozinger et al., 2003). As a releaser pheromone, it elicits worker attraction to the queen in the form of a retinue response, in which workers surround, lick, and antennate the queen (Keeling et al., 2003). Queen pheromone also helps to coordinate swarming (Winston et al., 1989). Finally, some components of the pheromone are used to attract drones to virgin queens during mating flights (Brockmann et al., 2006). Thus, components of this pheromone can act both as a sex pheromone and a social regulator.

The queens of the red imported fire ants, *Solenopsis invicta*, produce a pheromone that functions similarly to honey bee queen pheromone, eliciting both primer and releaser responses. It is synthesized in the postpharyngeal glands and the poison sac (Vargo and Hulsey, 2000). As a primer pheromone, it prevents virgin queens from shedding wings and activating ovaries (Vargo, 1998), and prevents mated queens from maturing and producing eggs (Fletcher and Blum, 1981). It also affects caste determination of female larvae (Vargo and Fletcher, 1986a,b, 1987), and it may alter aggression levels of workers toward nestmates and acceptance of newly mated queens (Klobuchar and Deslippe, 2002; Vander Meer and Alonso, 2002). As a releaser, it elicits worker attraction to the queen and induces workers to groom and feed the queen (Vander Meer et al., 1980). Three compounds have been identified that elicit this behavioral (releaser) response (Glancey et al., 1984).

In the termite *Reticulitermes speratus,* two chemicals that affect the reproductive division of labor recently have been identified (Matsuura et al., 2010). These two volatile

compounds, *n*-butyl-*n*-butyrate and 2-methyl-1-butanol, are produced by female neotenics (secondary queens) and suppress the development of additional neotenics within the colony. They also are detected on eggs in a colony. Similarly, in the higher termite, *Zootermopsis nevadensis,* four polyunsaturated alkenes (CHCs) have been associated with reproductive status of both males and females (Liebig et al., 2009).

Evidence for Control vs. Cooperation How these pheromones evolved to resolve reproductive conflicts between workers and queens has been long debated (Seeley, 1989; Keller and Nonacs, 1993; Gadagkar, 1997; Le Conte and Hefetz, 2008; Heinze and d'Ettorre, 2009). Traditionally, two main scenarios have been considered: the 'control' and the 'cooperation' scenario. The 'control' scenario proposes that queen pheromones manipulate workers to prevent them from activating ovaries in situations in which there is queen-worker reproductive conflict. Under this scenario, both the queen and the group benefit because the resources produced by the colony are used to support the reproductive output of the highly fecund queen. However, there is a cost to the individual fitness of workers, since they can compete with the queen to lay (usually male) eggs (Keller and Nonacs, 1993; Gadagkar, 1997). As a result, it has been argued that a 'control' scenario should create inherent conflict between queens and workers, and that it would be evolutionarily unstable and therefore unlikely to be maintained (Keller and Nonacs, 1993).

An alternative model has also been posited (Keller and Nonacs, 1993; Zahavi, 1993; Gadagkar, 1997; Heinze and d'Ettorre, 2009): queen pheromones could serve as an honest signal of the queen's fecundity to encourage worker 'cooperation.' In this case, worker inclusive fitness is increased by cooperation with a healthy, highly fecund queen (Gadagkar, 1997), but the queen signal is linked to reproductive state, allowing workers to detect failing or absent queens and initiate egg-laying in conditions most favorable to their inclusive fitness (Keller and Nonacs, 1993). This situation would reflect an evolutionarily stable strategy (e.g., a strategy that is resistant to cheating or invasion over time) because the interests of queens and workers are aligned. Note that honest signals are hypothesized to exert a cost to the producer, while reliable signals are simply correlated with a specific trait. We will refer to queen pheromone as an honest signal, consistent with previous discussions, even though only a handful of studies have demonstrated a cost to production of these pheromones (see discussion below).

Many subsequent studies have attempted to resolve the 'honest signal' vs. 'control' debate (see Table 1). However, it is important to note that the control and honest signal scenarios can be difficult to disentangle in advanced eusocial species because in some cases both scenarios may result in similar evolutionary outcomes. For example, Keller and Nonacs (1993) predicted a control scenario should result in a chemical arms race between queens and subordinates, and would result in a large number of novel components being incorporated into the pheromone and/or an increasing quantity of pheromone being produced. However, a sophisticated honest signaling system should also require a similarly elaborate system of chemical signals that perhaps are related to different aspects of a queen's physiological state. Thus, differentiating the ultimate causes of pheromone complexity is not possible *post hoc* (see also Le Conte and Hefetz, 2008; Keller, 2009; Alaux et al., 2010). Recognizing this, we outline recent studies that have examined these questions, and also highlight some of the caveats related to their findings.

As previously mentioned, one prediction from a control scenario is that a chemical arms race between queens and workers should result in a highly complex pheromone blend. In honey bees, pheromone signaling by the queen includes a large number of compounds produced by at least three glands (reviewed in Katzav-Gozansky, 2006). Furthermore, the chemical blends produced by the individual glands can have overlapping regulatory functions. For example, both mandibular glands and tergite glands elicit a retinue response and inhibit ovary activation even though there are no common chemicals (Slessor et al., 1988; De Hazan et al., 1989; Wossler and Crewe, 1999a, b; Hoover et al., 2003). However, evolution of such a complex chemical blend also is predicted by an honest signaling scenario, as noted above. Interestingly, honey bee queens have evolved a mechanism to bypass the olfactory system and directly alter worker neurophysiological processes. One of the main components of QMP (HVA) acts as a dopamine agonist in workers (Beggs et al., 2007; Vergoz et al., 2007; Beggs and Mercer, 2009). Moreover, treatment of queenless workers with dopamine enhances ovarian development in workers (Dombroski et al., 2003), suggesting that HVA may inhibit ovarian activation by acting agonistically on the dopamine pathway (Beggs et al., 2007; Beggs and Mercer, 2009), and thus providing evidence for queen control. However, the direct effects of HVA on ovarian activation of workers remain to be tested explicitly (Keller, 2009), with full evidence for queen manipulation requiring demonstration that worker fitness is decreased by the manipulation. Furthermore, if HVA did evolve to co-opt an existing neurophysiological system, the system should be conserved in species of *Apis* in which HVA is not used as a pheromone signal.

Keller and Nonacs (1993) also predicted that workers would likely be under strong selection to escape from the queen's control, perhaps by developing a tolerance or immunity to the pheromone. There is evidence for this in

Table 1 Evidence for signal vs. control signal scenarios in social insects

Scenario	Evidence	Species	References
Honest	Cuticular hydrocarbon profiles differ between egg-layers and non egg-layers	*Polistes dominulus, P. fuscatus, Vespa crabro, Bombus hypnorum, Dinoponera quadriceps, Streblognathus peetersi, Diacamma ceylonense, Harpegnathos saltator, Pachycondyla inversa, Platythyrea punctata, Myrmecia gulosa, Leptothorax acervorum, L. gredleri, Cataglyphis iberica, Camponotus floridanus, C. vagus, Formica fusca,* Linepithema *humile, Lasius niger*	Bonavita-Cougourdan et al., 1991; Sledge and Boscaro 2001; Dapporto et al., 2005; Espelie et al. 1994; Butts et al., 1995; Ayasse et al., 1995; Monnin and Peeters 1998; Peeters et al., 1999; Cuvillier-Hot et al., 2004a; 2004b; Cuvillier-Hot et al. 2001; Tsuji et al., 1999; Liebig et al., 2000; Heinze et al., 2002; Hartmann et al., 2005; Dietemann et al., 2003; Tentschert et al., 2002; Dahbi & Lenoir 1998; Endler et al., 2004; Hannonen et al., 2002; de Biseau et al., 2004 ; Holman et al., 2010a
	Dufour's gland composition is correlated with ovary activation in queens and workers	*Apis mellifera*	Dor et al., 2005; Malka et al., 2007; 2009b.
	Some components of mandibular glands are correlated with insemination volume and ovary activation in queens	*Apis mellifera*	Kocher et al., 2008; 2010a; Richard et al., 2007
	There may be a cost associated with pheromone production	*Lasius niger*	Holman et al., 2010b
	Reproductively active workers revert back to sterility when the queen is reintroduced	*Bombus terrestris; Apis mellifera*	Alaux et al., 2007; Malka et al., 2007
Control	Large number of compounds in pheromonal blend and multiple glandular sources could be indicative of an arms-race	*Apis mellifera; Solenopsis invicta*	Katzav-Gozansky et al. 2002; 2003; 2006; Vargo and Hulsey 2000
	4-hydroxy-3-methyoxyphenylethanol acts as a dopamine agonist to block aversive learning & memory	*Apis mellifera*	Beggs et al., 2007; Vergoz et al., 2007
	Workers should attempt to escape queen control	*Apis mellifera*	Moritz et al., 2002; Kocher et al., 2010b

honey bees, in which there are examples of modified worker response to QMP. In one honey bee strain (the 'anarchistic' bees), there appears to be a modification of worker physiological responses to pheromone (workers activate ovaries in the presence of a queen) rather than a change in worker attraction to pheromone or in queen pheromone production (Hoover et al., 2005). In a second strain, *Apis mellifera capensis*, workers with high reproductive potential are less attracted to the queen, potentially reducing their exposure to queen pheromone (Moritz et al., 2002). Interestingly, these bees and a few additional ant species are capable of thelytokous parthenogenesis; reproductive division of labor among workers in these species also may be maintained by pheromones that are correlated with individual fecundity (Monnin 2006, Heinze and D'Ettorre 2009).

Recent work has demonstrated that there is substantial variation in individual worker responses to queen pheromone within colonies of unselected European honey bees, and that workers within a colony with the highest reproductive potential are the least attracted to QMP (Kocher et al., 2010b). This variation in worker pheromone response implies that some workers are attempting to 'escape' queen pheromone. However, as queen pheromone is spread rapidly through the colony by workers (Naumann et al., 1991), it remains to be determined whether queen avoidance is sufficient for reducing the effects of queen pheromone. Furthermore, individual differences in response thresholds to social cues can optimize colony performance; variation in traits such as the initial onset of foraging or sucrose response may increase colony productivity and success (Beshers and Fewell, 2001; Oldroyd and Fewell, 2007, 2008). Similarly, optimization of colony performance could explain the extent of variation in worker responses to queen pheromone rather than evolutionary pressure to 'escape' queen control.

It also has been predicted that if queen pheromones act as honest signals, they should show an association with queen fecundity or mating number. While this association is not necessarily obligatory because workers could potential-

ly assess the level of eggs and brood throughout a queen's life, the majority of studies have demonstrated that queen-specific chemical blends are indeed associated with dominance or fecundity. Several studies of CHCs in ants, wasps, and termites (see above), have demonstrated that these chemical profiles act as pheromones, and are strongly correlated with ovarian activation level and with aggression in primitively eusocial species. Honey bee queen pheromone appears to be linked with ovarian activation (Kocher et al., 2008, 2009), and workers preferentially perform the retinue response on extracts from queens with the greatest amount of ovarian development (Kocher et al., 2009). Furthermore, insemination quantity affects queen mandibular gland composition, as well as its attractiveness to workers (Richard et al., 2007; Richard, F. J., Tarpy, D. R., and Grozinger, C. M., unpublished data). However, comparisons of virgin, drone-laying, and naturally mated queens, did not reveal differences in relative abundances of the six major compounds in the glands, although there did appear to be some shifts in absolute quantities of several of the main QMP components produced, including HVA (Strauss et al., 2008). Instead, minor components in the glands change in relative abundances and, therefore, may be key components associated with mating quantity/quality in queens and also presumably involved in altered worker retinue responses (Richard et al., 2007; Strauss et al., 2008; Kocher et al., 2009). Variation in inhibitory effects of different blends on worker ovarian development, however, remains to be demonstrated.

Studies in pheromone signaling among groups of queenless workers also support a link between ovarian activation and production of queen pheromones. Honey bee workers with activated ovaries will produce queen-like pheromone signals and engage in pheromonal contests, although laying workers produce much lower quantities of these compounds than do queens (Crewe and Velthuis, 1980; Plettner et al., 1993; Katzav-Gozansky et al., 1997; Moritz et al., 2000, 2004; Dor et al., 2005). In the initial stages of signal production, workers increase the levels of queen-like chemicals produced in mandibular glands and as ovarian activation continues, Dufour's glands secretions also begin to mimic the queen's signals. Mandibular gland secretions appear to be used to establish dominance rankings in the early stages of conflict, while the Dufour's glands appear to be strongly linked to worker ovarian activation (Malka et al., 2008). Notably, reproductively active, queenless honey bee workers (and bumble bee workers) revert back to sterility when the queen is reintroduced (Alaux et al., 2007; Malka et al., 2007) suggesting that an honest signaling mechanism is indeed at work (they do not compete with a fecund queen). However, these studies remain correlational; the exact mechanisms that link pheromone production to ovarian activation remain to be determined (but see Malka et al., 2007, 2009b).

There is also surprisingly little known about the energetic costs of pheromone production (Heinze and d'Ettorre, 2009). There is some evidence of a cost associated with pheromone production in honey bee queens in which the output of queen pheromone is thought to decrease in failing queens; however, this needs to be proven conclusively (Winston, 1987). In fact, in queens infected by *Nosema microsporidia*, quantities of queen pheromone increase (Alaux et al., 2011). On the other hand, recent work has demonstrated that *Lasius niger* queens that are immuno-compromised show a decrease in production of 3-methylentriacontane (Holman et al., 2010b), suggesting that there may be a cost associated with pheromone production at least in this species. Finally, reproductively active honey bee workers (Malka et al., 2008), bumblebee workers (Amsalem et al., 2009; Amsalem and Hefetz, 2010), and workers of the ant, *Aphaenogaster cockerelli* (Smith et al., 2009), produce queen-like pheromone blends and are more likely to be attacked by other workers also supporting a cost to production of pheromone.

The Evolution of Pheromonal Signals Examining queen pheromone production and response in an evolutionary context should improve our understanding of how these types of systems evolved in social insects. It has been suggested that pheromone signals evolved from systems regulating more basal pheromone-mediated behaviors, such as mating and defense, which are found even in solitary lineages of wasps (Blum, 1969). In fact, the main component (9-ODA) of honey bee queen pheromone also is used as a sex pheromone to attract males. However, comparative analyses of the compounds that mediate these activities in solitary species with the compounds that regulate division of labor in social species have yet to be produced. These types of studies have the potential to improve our understanding of how some of these basal pathways have led to the evolution of complex communication systems in social insects.

Analysis of queen pheromones of primitively eusocial species provides important insights into the evolutionary origins of queen pheromones. The primitive systems studied seem to possess simple signals that are linked to fecundity, indicating that early pheromones may have evolved from cues associated with fecundity into honest signals that were reinforced by aggression (see above discussion). As societies increase in complexity, greater caste-differentiation should reduce conflict between queens and workers, and pheromone signals may experience a decrease in selective pressures to maintain costs associated with honesty perhaps evolving into simple signals of queen presence. With increased caste differentiation and functional sterility of workers, the reproductive potentials of queens and workers should be disproportionate, and individuals

would no longer benefit from deceiving nestmates. In this situation, low-cost signals ('cheap-talk') are predicted to evolve (Crawford and Sobel, 1982; Bergstrom and Lachmann, 1998). Because a low level of intracolonial conflict is always maintained, these low-cost signals could be added on to the main pheromone component as reinforcement. As a result, we predict that as social complexity increases, pheromone signals should increase in complexity, associated with either (a) reinforcement of honesty via costs or other mechanisms, or (b) increased arms races between queens and workers. However, it is important to note that other mechanisms, such as worker policing, may also help to reduce conflict in the colony (Ratnieks and Visscher, 1989).

There is some evidence to support these predictions in the honey bee queen pheromone. This blend is highly complex, and includes both stable components (those that do not change in absolute or relative quantities with changes in fecundity) as well as variable components (those that vary with changes in fecundity). It is possible that the stable components of these blends may be evolutionary remnants of the low-cost signals, predicted in ancestral monogamous societies to signal queen presence but not necessarily fecundity. In turn, variable components may be the result of queen-worker conflict, and could vary according to the queen's physiological state and fecundity. Thus, we predict that major components of the blend would be associated with caste differences throughout the phylogeny, and would be relatively conserved across species, while minor components would vary according to the queen's physiological state and fecundity and would demonstrate a higher-degree of species specificity. Studies across the *Apis* phylogeny have demonstrated that major components of queen mandibular pheromone (the acids, 9-ODA, 9-HDA) are conserved across species while less abundant phenolics (HVA and HOB) have been found only in cavity-nesting species (Plettner et al., 1997). The acids are found in workers in all species, while HOB is found at very low levels in laying *Apis mellifera* workers, and HVA is not detected (Plettner et al 1993). These results suggest that different biosynthetic pathways evolved independently and potentially have different functions.

Examination of pheromonal blends across a phylogeny is surprisingly uncommon. Aside from the aforementioned studies in *Apis*, the evolution of pheromone signals in bark beetles and fruit flies also have been examined. In these studies, pheromone composition was superimposed onto a phylogeny to examine the evolutionary history of chemical components to infer the mode of evolution (Symonds and Elgar, 2004, 2008; Symonds and Wertheim, 2005). These studies suggest that in bark beetles, aggregation pheromones evolved through large, saltational shifts in composition, while in fruit flies, composition seems to have evolved more gradually, with closely-related species showing greater compositional similarity than divergent species. Similarly, rapid saltational changes of sex pheromones are associated with speciation in *Bactrocera* flies, followed by gradual divergence thereafter (Symonds et al., 2009). Most recently, the same group completed a study of patterns of worker CHC evolution across 56 ant species, and found that these compounds seem to evolve via gradual changes in pheromone composition as opposed to rapid, saltational shifts (van Wilgenburg et al., 2011). A similar, comparative approach could be used to study chemical communication systems in socially-variable lineages, such as halictid and allodapine bees in which life histories span the full range from solitary to eusocial among closely-related species (see Schwarz et al., 2007, for a detailed review of these systems).

Future Applications: Genomics as a Tool for Studying Chemical Communication Previous studies of chemical communication systems in social insects have relied almost exclusively on classical chemical ecology approaches. However, it is now possible to identify genes associated with pheromone production and perception and to study the evolution of pheromone communication systems at the molecular level using large-scale comparative studies across species. Substantially decreased sequencing costs enable transcriptome or whole genome sequencing of a number of Hymenoptera, including bee species (HGSC, 2006; Sadd et al., 2010; Woodard et al., 2011), paper wasps (*P. metricus*, Toth et al., 2007), three species of parasitic wasps (Werren et al., 2010), and six ant species (Bonasio et al., 2010; Smith et al., 2011a, b; Suen et al., 2011; Wurm et al., 2011). Genome projects are underway for other ant, bee, and wasp species. Genomic tools can be used to identify key genes or gene families (e.g., pheromone receptors) involved in chemical communication. There are several approaches to search for these genes. In some cases, it is possible to search for candidate genes that are homologous to known genes of a similar function in other species. For example, genes likely associated with queen pheromone biosynthesis in honey bees were identified based on sequence similarity to insect cytochrome P450 enzymes (Malka et al., 2009a).

In other cases, homologous genes may not have been identified, or these genes evolve so rapidly (such as olfactory receptors) that it is impossible to predict which ones are involved in detection of pheromones vs. other chemical cues. In such cases, finding candidate genes can be accelerated by monitoring expression patterns associated with a behavioral or physiological state using either microarray technology (Gibson 2003) or RNA-sequencing (RNA-seq; Wang et al. 2009). For example, the honey bee

olfactory receptor, AmOR11, was identified as the receptor for 9-ODA (the major component in honey bee queen pheromone) by using microarrays and subsequently verified by an *in vitro* approach (Wanner et al., 2007). Furthermore, microarray studies of brain gene expression demonstrated that queen mandibular pheromone, brood pheromone, and alarm pheromone alter worker-brain gene expression patterns in a manner consistent with their effects on behavioral maturation (Grozinger et al., 2003; Alaux et al., 2009), suggesting that specific pheromone-responsive gene networks have evolved in the brain to translate pheromonal cues into behavioral changes. Finally, microarray studies have identified expression differences associated with variation in responsiveness to queen mandibular pheromone (Kocher et al., 2010a; Fussnecker et al. in press). These studies were made possible by the completion of the honey bee genome (HGSC, 2006) and development of a genome-wide honey bee microarray. RNA-seq enables the search for transcriptional differences among individuals in any organism, regardless of genome availability and, thus, we expect studies similar to those completed in *Apis mellifera* to become commonplace. This should enable a number of evolutionary questions to be addressed. For example, is regulation of social hierarchies by aggression in primitive species similar to pheromonal regulation of these hierarchies in advanced species?

Transcriptional studies can give some indication of the physiological mechanisms that underlie pheromone production and response and also identify genetic variants involved in chemical communication. These must be done in conjunction with genetic mapping or functional studies that seek to identify causal variants. Traditionally, mapping studies have been limited by an ability to identify a large number of randomly-distributed genetic markers; methods such as microsatellites or AFLPs were too labor intensive to produce. Now, development of microsatellite markers can be completed by using high-throughput technology, such as 454, and completed quickly and efficiently using less than a full run on a 454 machine (Abdelkrim et al., 2009). Furthermore, sequencing of parental colonies of crosses used in linkage mapping also can be used to identify a large number of single nucleotide polymorphisms (SNPs) specific to the base population, and a number of genotyping platforms can be employed to characterize these high-density markers in subsequent generations of the mapping population. Thus, identification and genotyping of a large number of genetic markers is no longer rate limiting, and mapping studies can be completed efficiently in any organism that is easy to rear in the laboratory and which can be readily crossed. Despite these advances, mapping studies for traits associated with chemical communication are surprisingly uncommon. One study identified a single QTL containing four odorant receptors that underlie the perception of reproductively isolating sex pheromones of two *Heliothis* moths, *H. subflexa* and *H. virescens*; the functional importance of the region was confirmed through a combination of behavioral and neurophysiological assays (Gould et al., 2010). Another study identified fixed genetic differences associated with differences in CHC production in males of *Nasonia giraulti* and *Nasonia vitripennis* (Niehuis et al., 2010).

It is important to note that in many non-model organisms, laboratory crosses are not readily produced, liniting the ability to map critical genetic variants. Recently, the field of population genomics has enabled association-mapping studies in natural populations without the need for complex crossing schemes (Stinchcombe and Hoekstra, 2008). Using these tools, one can now address questions of genetic relatedness using the recombination generated in natural, field populations. There are several recent reviews that summarize these approaches (Stinchcombe and Hoekstra, 2008; Nadeau and Jiggins, 2010; Stapley et al., 2010).

Molecular evolutionary approaches can be used not only to identify conserved genes across species, but also to characterize the evolutionary forces affecting these genes. For example, searching for genes that show strong signatures of stabilizing selection within a species, but with high divergence between species that are reproductively isolated via chemical communication, could identify candidate genes underlying pheromonal signaling in the respective species. Furthermore, expansion of these comparative studies across multiple species can determine whether the genes and gene pathways regulating pheromone production, detection, or responses to pheromones are conserved or not. A molecular evolutionary approach then can be applied to characterize the patterns of selection on these genes. By placing these genes in a phylogenetic context, one can test theories about how these pheromones evolve.

Following the identification of candidate loci, functional genomic approaches can be used to characterize these regions via transcriptional studies, mapping approaches, and/or comparative genomic studies. These approaches include RNAi, transgenics, or the use of other functional genomic resources available in traditional genetic model systems (e.g., *Drosophila*; Belles, 2010). For example, several candidate moth pheromone receptors were validated by using heterologous expression systems in *Drosophila melanogaster* to identify or confirm putative ligands (Syed et al., 2006).

The combination of powerful genomic technologies and functional testing of candidate genes promises to improve our understanding of the molecular pathways that underlie pheromone production and response. Moreover, the increased accessibility of these tools will enable studies on the molecular basis of chemical communication in a wider range of taxa.

Analysis of these data within a phylogenetic context will facilitate determination of whether similar genes are involved in regulating chemical communication across species and how far the conservation of these mechanisms persists across evolutionary history. For example, do similar genes regulate chemical communication across all ants or only among closely related species? Are some molecular pathways highly conserved while others are more variable within and between species? Finally, molecular evolutionary approaches will help elucidate the selective forces that have played a role in shaping chemical communication systems.

Conclusions

Elucidation of the factors that led to the evolution of complex chemical communication systems is challenging: it requires characterization of both proximate and ultimate factors underlying pheromone production and response. Recent studies have improved our understanding on both levels. Based on the available data, it appears that honest signals are important in the origin and elaboration of queen pheromone blends. However, we hypothesize that later pheromone blends evolved to have both caste-specific components and components that vary according to reproductive state and health. We have outlined a number of functional approaches that should help elucidate the molecular mechanisms underlying signal production and response, as well as comparative approaches that may be used to dissect some of the ultimate factors leading to these chemical repertoires. Through the combination of these complementary approaches, it should be possible to place these mechanisms in a broader phylogenetic context to understand better the evolutionary history of pheromone-mediated communication.

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2002; Shimada and Saitoh 2006). The countermeasure used by the large Japanese wood mouse *Apodemus speciosus* against acorn tannins has been elucidated (Shimada et al. 2006). Captive wood mice could be acclimated to ingest gradually increasing amounts of tannin, whereas those without acclimation suffered severe damage. Production of tannin-binding salivary proteins and increased activity of tannase-producing enterobacteria were the likely mechanisms of acclimation (Shimada et al. 2006).

Proline-rich proteins (PRPs) are the prevalent types of tannin-binding salivary protein and are produced by the Japanese wood mouse (Shimada et al. 2004). As their name suggests, proline comprises about 20–40% of the amino acids in PRPs (Mehansho et al. 1987; Shimada 2006). PRPs have a high affinity for tannins and are recognized as the first line of defense against tannins in some mammals (Hagerman and Robbins 1993; McArthur et al. 1995). PRPs form stable complexes with tannins in the oral cavity, preventing tannins from interacting with other proteins and from degrading into noxious phenolic compounds (Hagerman and Robbins 1993; Skopec et al. 2004; Shimada 2006). Tannin–PRP complexes are stable in the gastrointestinal tract, and are excreted in the feces (Mitaru et al. 1984; Skopec et al. 2004).

Since some animals can acclimate to tannins, the evaluation of prior exposure is the first step to understanding tannin tolerance in a target species. However, it is difficult to quantitatively estimate tannin intake in wild animals, especially in nocturnal small mammals. Their feeding behaviors are difficult to observe in the field and analysis of stomach or fecal contents cannot provide sufficient information on food items at the species level (Hansson 1970; Tatsukawa and Murakami, 1976; Henry 1999). Therefore, we proposed to develop a new method for estimating tannin intake by wild animals by focusing on the proline content of feces as an index of tannin intake.

The first aim of our study was to develop a method for estimating tannin intake in small, free-living rodents. To achieve this, we examined the relationships between dietary tannin content and fecal proline in a feeding experiment conducted with three sympatric forest-dwelling rodent species, the large Japanese wood mouse *A. speciosus*, the small Japanese wood mouse *A. argenteus*, and the gray-sided vole *M. rufocanus*. We applied this method to the three rodent species in a natural forest in Hokkaido, in the northernmost part of Japan, and we revealed interspecific differences in seasonal changes in tannin intake.

Methods and Materials

Rodent Species The two species of *Apodemus* are endemic to Japan, and *M. rufocanus* is distributed widely from Fennoscandia to eastern Asia. These three species are sympatric in Hokkaido (Ohdachi et al. 2009). The two *Apodemus* species are typical forest-dwelling species, whereas *M. rufocanus* is common in both forests and open habitats (Ohdachi et al. 2009). Adult body mass ranges from 20 to 60 g in *A. speciosus*, 10 to 20 g in *A. argenteus*, and 27 to 50 g in *M. rufocanus* (Ohdachi et al. 2009).

Animals Used *Apodemus speciosus* and *A. argenteus* used in the following experiments were caught in October 2007 in the Nakaimura national forest (Iwate Prefecture, northern Honshu 39°81′N, 141°57′E) and *M. rufocanus* individuals were caught in July 2009 in the Uryu Experimental Forest of Hokkaido University (44°03′N to 44°29′N, 142°10′E to 142°20′E). They were weighed, housed separately in plastic cages with paper bedding (Alpha-dri, Shepherd), and allowed free access to distilled water and food, consisting of laboratory chow (MM-3 designed for laboratory mice and rats, Funabashi Farms) and oats, under regulated environmental conditions (20°C; photoperiod, 12 hL: 12 D) until the beginning of the feeding experiments. All procedures in the field and laboratory were approved by the Animal Experimentation Committee of the Forestry and Forest Products Research Institute.

Feeding Experiments In order to examine the relationships between dietary tannin and fecal proline, we performed feeding experiments using a powdered formula diet that had a nutritional composition similar to that of acorns of *Quercus crispula* and *Quercus serrata* in terms of all major constituents except tannins (hereafter referred to as the basal diet; see details in the work of Shimada and Saitoh 2003). Tannic acid (Wako Pure Chemical Industries) was added to the basal diet at 1, 2, 3, or 4% on a dry weight basis (hereafter referred to as the 1–4% TA diets). These diets were supplied in sequence from the basal diet to the 4% TA diet to 7 animals of *A. speciosus* (April 2008), 5 of *A. argenteus* (April 2008), and 9 of *M. rufocanus* (August 2009). Because the tannic acid was well blended with the basal diet, test animals could not avoid the tannic acid, and the dietary tannin content was assumed to be the tannin intake of the rodents. Each diet was supplied for 5 d; to avoid carryover effects of the prior diet, only the feces excreted during the last 2 d were collected for further analysis.

After the conclusion of the TA trial, all of the mice and 6 of the 9 voles were fed the basal diet for 7 d, hereafter referred to as the tannin-free period. During this period, all feces were collected daily to determine the baseline proline content, namely the fecal proline level when rodents were not ingesting tannin

Estimating Tannin Intake in the Field We tested the method on the three rodent species in their natural habitat. The investigation was performed in the Uryu Experimental Forest, which is located in a cool temperate wet climate zone. The yearly average temperature is 3.5°C, annual precipitation is 1,410 mm, and maximum snow depth is 275 cm. The study site was a natural forest of broadleaf trees and conifers. The dominant tree species were *Acer mono*, *Q. crispula*, *Betula ermanii*, *Sorbus commixta*, *Abies sachalinensis*, and *Picea glehnii.* The undergrowth was dominated by dwarf bamboos, *Sasa senanensis* and *Sasa kurilensis*.

Live trapping was performed for three consecutive days once a month from June to October 2008 using Sherman-type live traps. Species, sex, body weight, and reproductive status were recorded upon capture. In addition, feces left in the trap were collected in a plastic bag and stored in a freezer. Each trap was cleaned with water before and after the trapping survey. Some of the captured rodents were transferred to the laboratory, housed separately at a constant temperature (24°C) in plastic cages with paper bedding, and allowed free access to distilled water and the basal diet. For estimating tannin intake, it is necessary to adjust the regression equations for fecal proline to dietary tannin using the baseline fecal proline content (see results). Thus, all feces from the rodents transferred to the laboratory were collected daily for 7 d after rodent capture to determine the baseline.

Chemical Analyses All chemicals used were of either analytical grade or the highest purity grade available. All feces were freeze-dried, milled with a mortar and pestle, and stored in a desiccator until analyzed.

Proline contents were estimated by using the method of Skopec et al. (2004). Briefly, samples (about 20 mg) were hydrolyzed at 110°C for 20 h under nitrogen with 400 μl of 6 N HCl containing 1% phenol. After hydrolysis, the contents were filtered and diluted to 20 ml with distilled water. To remove HCl, 200 μl of the sample were evaporated, the residue was dissolved with 100 μl of distilled water, re-evaporated, and redissolved in 200 μl of distilled water. A 100 μl aliquot was mixed with 400 μl of 1% ninhydrin solution, incubated at 100°C for 1 h, cooled and analyzed at 520 nm using a microplate reader. The method was standardized with a series of standard proline solutions (0–200 μmol/l) and concentrations of proline were expressed on a dry weight basis.

Data Analyses To examine the relationship between fecal proline content and dietary tannin content, linear mixed model regression analyses were conducted; proline in fecal contents was regressed against dietary tannins, and individual rodent ID was considered as a random effect. The 95% confidence and prediction limits also were estimated. To examine the pattern of decline of fecal proline content, linear mixed model ANOVA (with individual rodent ID as a random effect) was performed. Multiple comparisons were conducted with Tukey-Kramer's HSD test.

Tannin intake in the field was estimated by using the regression equations obtained in the feeding experiment. The regression equations were adjusted in accordance with the baselines estimated for each species and for each month. Differences in these baselines were examined by two-way ANOVA (with species and month as fixed effects). Seasonal changes in fecal proline and tannin intake in the three rodent species captured at the study site were analyzed with one-way ANOVA, and multiple comparisons were conducted with Tukey-Kramer's HSD test. All statistical analyses were conducted with the JMP 8 statistical package.

Results

Relationship between Dietary Tannins and Fecal Proline Linear mixed model regression analyses revealed significant positive relationships between dietary tannins and fecal proline in all three rodent species (Table 1 and Fig. 1). The coefficients of determination were high in the two *Apodemus* species (*A. speciosus*, R^2=0.791; *A. argenteus*, R^2=0.888). Unlike in the two *Apodemus* species, the fecal proline content in *M. rufocanus* was slightly lower with the 4% TA diet (mean ± SE, 0.73±0.08) than with the 3% TA diet (0.79±0.06). Therefore, we examined another model in which dietary tannin was limited to 3%, and all of the results obtained with the 4% TA diet were omitted from the analysis. The coefficient of determination was much higher in the limited proline model (R^2=0.885) than in the original proline model (R^2= 0.594).

Fecal proline content decreased in all three species after the supply of dietary tannins was stopped. The fecal proline content on day 1 of the tannin-free period was 13.7% lower than it was on the 4% TA diet in *A. speciosus*, 16.6% lower in *A. argenteus*, and 32.6% lower in *M. rufocanus* (Fig. 2). Fecal proline content reached a minimum by day 3 in *A. speciosus* and *A. argenteus*, and by day 1 in *M. rufocanus* (Fig. 2).

Regression Equations for Estimating Tannin Intake Based on the above results we chose the proline content on day 5 of the tannin-free period as the baseline for each species. Thus, the baselines (means ± SE) in this experiment were 0.57%±0.04% in *A. speciosus*, 0.69%±0.05% in *A. argenteus*,

Table 1 Summary of results of linear mixed model regression analyses

Species	No. of rodents	Response variable	*N*	R^2	Estimation of parameters (± SE)	
					Intercept	Dietary tannins (%)
Apodemus speciosus	7	Proline	35	0.791	0.68±0.08***	0.21±0.02***
Apodemus argenteus	5	Proline	25	0.888	0.84±0.07***	0.31±0.03***
Myodes rufocanus	9	Proline (Dietary tannin level: 0–4%)	45	0.594	0.46±0.05***	0.09±0.02***
		Proline (Dietary tannin level: 0–3%)	36	0.885	0.41±0.04***	0.14±0.01***

* $P<0.05$; ** $P<0.01$; *** $P<0.001$

and 0.34%±0.02% in *M. rufocanus.* The regression equations were adjusted as follows:

$$Pf' = 0.11+(0.21 \times Ti)(\text{for } A.\ speciosus)$$
$$Pf' = 0.16+(0.31 \times Ti)(\text{for } A.\ argenteus)$$
$$Pf' = 0.07+(0.14 \times Ti)(\text{for } M.\ rufocanus)$$

Pf' (%) denotes the difference between the observed fecal proline content (*Pf*) and the baseline, and *Ti* is the tannin intake (% TAE). Tannin intakes in the field were estimated according to these equations based on the observed *Pf* and baseline values.

Seasonal Changes in Estimated Tannin Intake The number of rodents caught in each month ranged from 0 to 10 for each species. No *A. speciosus* were captured in June. Fecal proline content in *A. speciosus* and *A. argenteus* increased in autumn (Fig. 3). This pattern was significant in both species (one-way ANOVA; *A. speciosus,* $F_{3,\ 15}=7.84$, $P<0.001$; *A. argenteus,* $F_{4,\ 24}=11.3$, $P<0.001$). Multiple comparisons also revealed that the fecal proline content in October was significantly higher than in the other months (Fig. 3). Fecal proline content in *M. rufocanus* also exhibited a seasonal change (one-way ANOVA, $F_{4,\ 34}=3.07$, $P=0.029$); the content was significantly but only slightly higher in October than in August (Fig. 3).

The baselines (i.e., fecal proline content on day 5 of feeding of the basal diet) differed significantly among species but not among months (two-way ANOVA; effect of species: $F_{2,\ 53}=13.8$, $P<0.001$; effect of month: $F_{4,\ 53}=0.989$, $P=0.422$; Table 2). Overall, these baselines were higher than those obtained in the feeding experiment, especially in *M. rufocanus*, in which the baseline fecal proline content in the field was even higher than the fecal proline content during feeding of the diet including tannins (Fig. 1c).

Estimated tannin intake varied significantly among seasons in the three rodent species (Fig. 3; one-way ANOVA; *A. speciosus,* $F_{3,\ 15}=17.1$, $P<0.001$; *A. argenteus,* $F_{4,\ 24}=8.93$, $P<0.001$; *M. rufocanus*, $F_{4,\ 34}=9.03$, $P<0.001$). Estimates in *A. speciosus* increased in September and October (Fig. 3a). Those in *A. argenteus* were almost zero from June to September and then increased significantly in October (Fig. 3b). Although estimated tannin intake in *M. rufocanus* fluctuated largely during the observation period, it was mostly below zero and did not show autumnal increase (Fig. 3c). Estimated tannin intake in *M. rufocanus* did not exceed 3% (the upper limit of the model), except in one case in October (3.46%).

Discussion

The feeding experiments demonstrated that tannin intake could be estimated from fecal proline content in the three rodent species examined. To determine the validity and limitations of this method for estimating tannin intake in the field, we investigated the mechanisms underlying the observed relationships.

Relationship between Fecal Proline and Tannin Intake The fate of tannins after their ingestion is not fully understood. Nonetheless, it is recognized that some tannin fractions are bound into insoluble complexes with proteins and other biopolymers in the gut, and some are metabolized by processes such as hydrolysis and glucuronidation (Waterman and Mole 1994; Makkar 2003). Consequently, the feces contain tannin–protein complexes, degraded phenolics, and various metabolic derivatives of tannins.

Tannin–PRP complexes are stable in the gut, and are excreted in the feces (Mitaru et al. 1984; Skopec et al. 2004). Although some of these complexes may be degraded by the action of tannase-producing enterobacteria (Shimada et al. 2006), this degradation affects the structure of the tannins and not the PRPs (Aguilar et al. 2007). Thus, it is likely that the feces contain most of

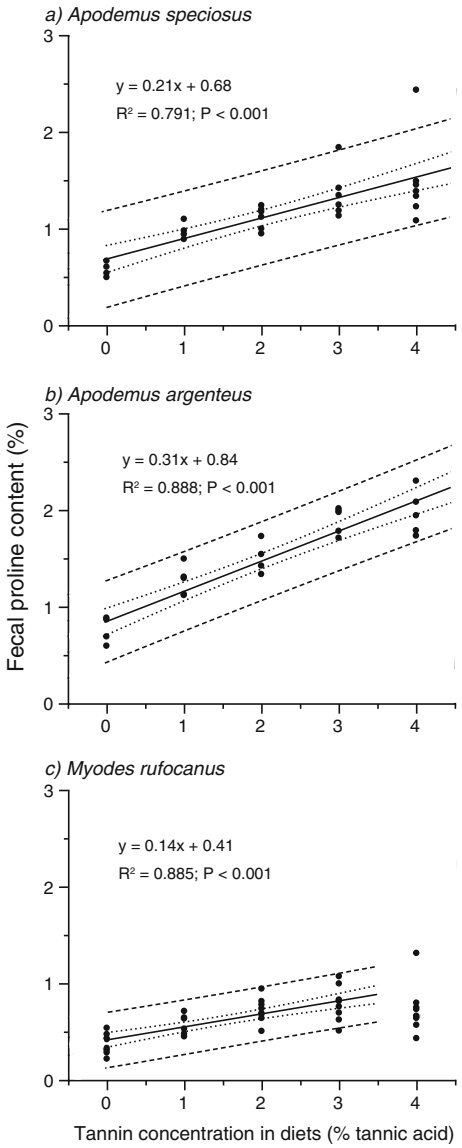


Fig. 1 Relationships between tannin concentration in the diet and fecal proline content in the three rodent species given a powdered formula diet including different levels of tannic acid: **a**) *Apodemus speciosus*, **b**) *Apodemus argenteus*, and **c**) *Myodes rufocanus*. Solid, dotted, and dashed lines represent the regression line, 95% confidence limits, and 95% prediction limits, respectively. The regression line for c) *M. rufocanus* was constructed by using only those results for 0% to 3% intake of tannin

the proline from the tannin–PRP complexes, and that there is a positive relationship between dietary tannin content and fecal proline. Provided that the animal produced enough PRPs to bind the dietary tannins, the amount of tannin–PRP complex would be proportional to the amount of tannins ingested.

Validity and Limitations of this Method This novel method of estimating tannin intake from fecal proline content is useful for several reasons. First, it has sufficiently high predictability for practical use, as shown by the coefficients of determination (Table 1). Second, this method allows us to estimate tannin intake only from the fecal concentration of proline. In other words, we do not need to know the total amount of proline excreted in a day or unit time, which would be difficult to estimate in a free-living animal. This point is especially useful in the field. Third, the method is noninvasive. Finally, considering the underlying mechanisms of this method, it may be applicable to a variety of mammals that induce PRPs to accommodate dietary tannin. The relationship between dietary tannin and fecal proline content would need to be examined in each species. Mammals that have been confirmed to produce PRPs include rodents, which can induce PRPs by ingesting tannins (Shimada 2006). Some species constitutively produce PRPs at high levels (Oppenheim et al. 1971; Austin et al. 1989). The method would not be useful in cases where PRPs are not induced by dietary tannin.

There are some limitations to this method. First, to obtain accurate estimations, feces collected from captured animals

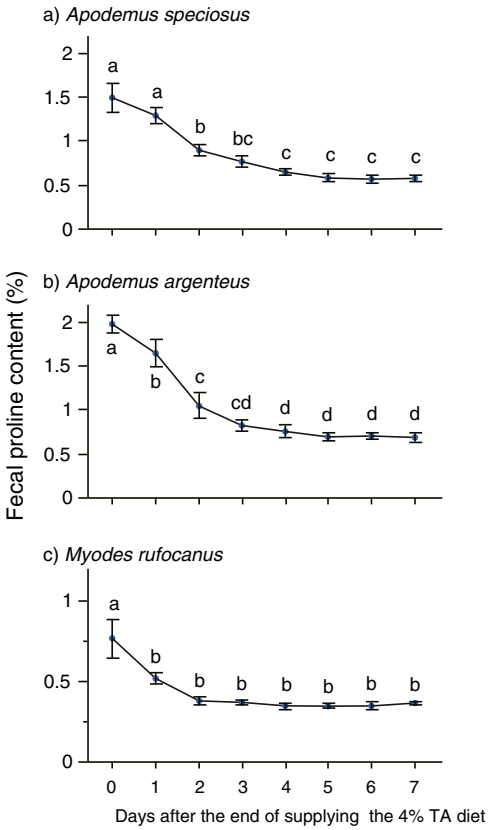


Fig. 2 Changes in fecal proline content (% ± SE) in the three rodent species: **a**) *Apodemus speciosus*, **b**) *Apodemus argenteus*, and **c**) *Myodes rufocanus*. Rodents were fed the basal diet for 7 days after the conclusion of the 4% TA (tannic acid) diet. Different letters indicate significant differences by Tukey-Kramer' s HSD test. Note that the scales on the vertical axes differ among the panels

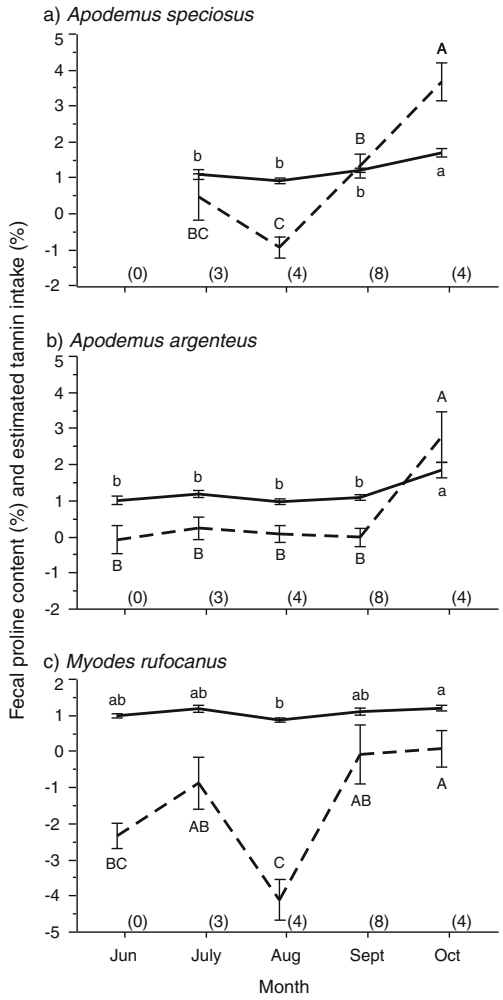


Fig. 3 Changes in fecal proline content (solid line,% ± SE) and tannin intake level estimated based on fecal proline content (dashed line,% TAE ± SE) in the three sympatric rodent species caught in the field (Uryu Experimental Forest, Hokkaido University): **a**) *Apodemus speciosus*, **b**) *Apodemus argenteus*, and **c**) *Myodes rufocanus*. Different letters indicate significant differences by Tukey-Kramer' s HSD test. Numerals in parentheses denote numbers of sample rodents

on laboratory diets should not be used to assess field diets. We found that fecal proline content decreased steeply after the animals were supplied with a tannin-free diet (Fig. 2). The second limitation is related to the baseline values of fecal proline. Ideally, the basal diet should yield a constant baseline level of proline. However, the values obtained with the basal diet varied not only among species but even within a species. Although we have no firm theories to explain this, it is possible that the long period of time that the animals were kept in the laboratory might have influenced the results. The intervals from capture to the feeding experiments were 6 months in the *Apodemus* species and 1 month in *M. rufocanus*. Assuming that fecal proline content could be influenced by physiological factors such as bacterial flora and metabolic status, these long intervals could have altered the synthesis or metabolism of proline. When this method is applied to free-living animals, it is necessary to measure the baseline in each case.

Finally, the validity of this method was confirmed only for tannic acid (a non-uniform mixture of gallotannins), but not for other types of tannins. On the basis of their chemical structures, tannins can be divided into two major groups: condensed and hydrolyzable (Yoshida et al. 2005). Hydrolyzable tannins are further divided into galloyl glucoses, gallotannins, and ellagitannins. These types of tannins differ in characteristics such as the ability to bind proteins (Mehansho et al. 1987), the sensitivity to hydrolysis (Waterman and Mole 1994), and the susceptibility to oxidation (Salminen and Karonen 2011). The regression equations relating fecal proline to dietary tannin would probably be different if another type of tannin were used as the standard. It clearly is important to compare the tannin intake level estimated by this method to tannin content and types of tannins in likely food plants when applying this method. Accordingly, the estimations would be more accurate if the tannins used as the standard were isolated from each of the main food plants for each herbivore.

Table 2 Baseline fecal proline contents (mean ± SE) in the three sympatric rodent species captured in Uryu Experimental Forest, Hokkaido University

Species	June	July	August	September	October	Mean ± SE
Apodemus speciosus		0.87	0.99±0.06	0.80±0.10	0.79±0.09	0.89±0.12 [a]
	(0)	(1)	(2)	(5)	(3)	(11)
Apodemus argenteus	0.89±0.06	0.94±0.06	0.78±0.04	0.92±0.21	0.82±0.06	0.88±0.09 [a]
	(3)	(5)	(4)	(4)	(5)	(21)
Myodes rufocanus	1.22±0.11	1.22±0.04	1.34±0.09	1.03±0.20	1.10±0.08	1.21±0.09 [b]
	(5)	(6)	(7)	(5)	(5)	(28)

Figures in parentheses represent numbers of rodents used for estimation of baselines. Different superscripts indicate significant differences by Tukey-Kramer' s HSD test.

Previous approaches to estimate tannin intake in free-living animals required two steps; first, to identify food plants of a target species by direct observation or analyses of stomach or fecal contents; and then to establish the amount of tannin contained in each food plant. However, it is difficult to quantitatively estimate tannin intake by this approach in small mammals, especially in nocturnal ones, because their feeding behaviors are hard to observe in detail in the field and food items from stomach or fecal contents can be hard to identify at the species level (Hansson 1970; Tatsukawa and Murakami, 1976; Henry 1999). Despite its limitations, the fecal proline method is an innovative tool for evaluating tannin intake in free-living animals that cannot be studied by more direct approaches.

Seasonal Changes in Tannin Intake in the Field Estimated tannin intake increased significantly in autumn for the two *Apodemus* species. The diets of the *Apodemus* species are predominantly granivorous and subdominantly insectivorous (Ohta et al. 1959; Tatsukawa and Murakami 1976). It is well documented that theses species forage heavily on acorns in autumn (Miyaki and Kikuzawa 1988; Takahashi et al. 2007), and that the study year, 2008, was a mast year for *Q. crispula*. The autumnal increase in tannin intake in *Apodemus* species may result from the mice eating acorns. This implies that acorns played an important role as sources of tannins in *A. speciosus* and *A. argenteus*, at least at this site in this year. *Myodes rufocanus* feeds mainly on green plants (Ohta et al. 1959; Ohta 1984). Contrary to the results of the *Apodemus* species, estimated tannin intake in *M. rufocanus* was close to zero throughout the year indicating that this species rarely ingests tannins in this habitat.

These findings are in good agreement with analyses of rodent population dynamics at this study site. In this area, the Field Science Centre of Hokkaido University has monitored the abundance of rodents and of acorns of *Q. crispula* since 1992, and Saitoh et al. (2007) examined the relationships between the population dynamics of the three species and acorn abundance. They found that the influence of acorn abundance on the population dynamics is large in *A. speciosus*, moderate in *A. argenteus*, and small or nonexistent in *M. rufocanus* (Saitoh et al. 2007). This consistency with field data further emphasizes the validity and usefulness of our novel method for estimating tannin intake in free-living rodents.

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of protein by half. However, both theoretical analysis (Moran and Hamilton, 1980) and experimental studies (Kuijper et al., 2004) have concluded that reduced protein digestibility is likely to cause compensatory feeding in small mammals (Meyer et al., 2010), and therefore, an increase in daily intake by herbivores. Compensatory feeding is likely in the case of ceacalids, such as the snowshoe hare, that rapidly pass plant biomass through the gut (Kuijper et al., 2004; Stott, 2008). Therefore, an alternative mechanism is needed to explain the negative effects of birch resin on intake by hares.

Hares may reduce intake to maintain concentrations of toxic PSMs below a deleterious threshold in the blood (Wiggins et al., 2003; Boyle et al., 2005; Sorensen et al., 2005; McLean and Duncan, 2006; Mclean et al., 2007; Torregrossa and Dearing, 2009). For example, in no-choice feeding trials with PA-rich CAG twigs of juvenile *B. neoalaskana*, food intake was reduced sufficiently that the hares would rapidly starve to death if an alternative, PA-free, food was not available (Reichardt et al., 1984).

Rigorous testing of the toxicity hypothesis requires knowledge of the presumed toxin's mode of action (MOA) and the concentration of PSM that elicits toxicity and ultimately reduces intake. The link between concentration (pharmacokinetics) and the MOA that elicits a response (pharmacodynamics) is at the heart of the field of pharmacology, and it is used to predict variable response of individuals and populations of humans to drugs (McLean and Duncan, 2006; Sorensen et al., 2006). Likewise, ecologists are typically interested in predicting the variable response of populations of herbivores to plants. In the case of birch and snowshoe hares, there is correlative evidence that populations of birch and hares are involved in a biochemical interaction that has resulted in a mosaic of co-evolution (Bryant et al., 1994, 2009; Swihart and Bryant, 2001). Yet, as is the case in the majority of studies on interactions between plants and vertebrate herbivores (Provenza et al., 1994; Provenza, 1995; Lawler et al., 1998; Forbey and Foley, 2009; McLean et al., 2009), the MOA for chemicals in birch has not been investigated in hares. We propose that pharmacological approaches that reveal the MOA of PSMs (Forbey and Foley, 2009; Sotka et al., 2009) will help ecologists scale up variation observed in plant chemistry and tolerance to PSMs by herbivores from individuals to populations.

Our objective was to use the birch-snowshoe hare system to identify the MOA of the resinous dammarane triterpene, papyriferic acid. This study builds on recent evidence that the MOA of PA in domestic rabbits, laboratory rats, and oxen involves uncompetitive inhibition of succinate dehydrogenase (SDH, McLean et al., 2009). Succinate dehydrogenase is part of both the citric acid cycle and respiratory electron transfer chain in mitochondria (Rutter et al., 2010). Inhibition of SDH interferes with cellular energy production and may be detrimental to health of animals (Wallace and Starkov, 2000). We tested our hypothesis that PA inhibits snowshoe hare SDH in a dose-dependent manner by measuring the effect of PA on the oxidation of succinate in mitochondria isolated from wild hares. In addition, we used molecular modeling to examine the hypothesis that PA inhibits SDH by binding to the ubiquinone sites on complex II.

Methods and Materials

Chemicals Papyriferic acid was prepared from the ether extract of the resin of CAG twigs of winter-dormant *B. neoalaskana* saplings (Reichardt, 1981). Authenticity of PA was confirmed by using ^{1}H NMR with a standard Triple Resonance Inverse probe (Bruker Biospin, Billerica, MA, USA) and Quadrupole Time of Flight mass spectrometry (Q-TOF, Bruker Daltonics, Billerica, MA, USA). The molecular mass (604.367 Dalton) and the ^{1}H spectral data were in agreement with data previously reported for PA (Reichardt, 1981). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of ACS grade.

Laboratory Animals Six-week old male Wistar rats and C57Bl/6 mice were purchased from Simonsen Laboratories (Gilroy, CA, USA). Animals were housed in polycarbonate cages and provided with an NIH-07 diet and water *ad libitum* for 7 d prior to tissue collection. Rats and mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Upon dissection of each euthanized animal, the liver was perfused with 10 ml ice cold 0.9% NaCl. Samples were snap-frozen in liquid nitrogen and stored at −80°C until use. The experiments were approved by the Boise State University Institutional Animal Use and Care Committee (006-AC10-002).

Snowshoe Hares Snowshoe hares were trapped near the Bonanza Creek Experimental Forest, approximately 20 km SW of Fairbanks, AK, USA (64° 41′ 37″ N, 148° 17′ 30″ W) using #3 Havahart live-traps baited with 300 g of fresh carrots and alfalfa cubes. The hares were sacrificed near the site of capture using cervical dislocation, and liver samples were collected immediately from each animal and snap-frozen in liquid nitrogen. Samples then were transported in a liquid nitrogen dry shipper and stored at −80°C until use. The hare experiments were approved by the Boise State University and University of Alaska-Fairbanks Institutional Animal Use and Care Committees (006-AC10-004 and 130970–5, respectively).

Mitochondria Preparation Mitochondria were prepared by thawing liver samples on ice and rinsing the tissue with cold 0.9% NaCl solution to remove blood. Tissues were cut into small pieces, homogenized in 5 volumes of isolation buffer containing 0.32 M sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4, and centrifuged for 10 min (1000×g, 4°C). Supernatants were transferred to clean vials and centrifuged for 15 min (12000xg, 4°C), followed by washing the pellets with isolation buffer and recentrifuging for 15 min (12000xg, 4°C). The final pellets from the same animal were resuspended in isolation buffer, pooled, and stored at −80°C until use.

Succinate Dehydrogenase (SDH) Activity Assay SDH activity was determined as described previously (Rosen et al., 1987; McLean et al., 2009) with adaptations for a 96-well format. Different concentrations of succinate (0–10 mM) were incubated with mitochondria to examine the enzyme kinetics of SDH with and without the addition of various concentrations of PA (10, 40, 80, and 160 μM). A single concentration of malonic acid (MA, 80 μM), a competitive inhibitor of SDH (Wojtovich and Brookes, 2008), was used as a control. In a 96 well plate, 5 μl of mitochondrial preparation were mixed with 5 μl of buffer containing the desired concentration of PA or MA and 220 μl of reaction media containing 2, 6-dichlorophenol indophenol (DCPIP) and sodium azide. The mixture was incubated for 5 min at 37°C before initiating the reactions by the addition of 20 μl of buffer that contained the desired concentration of succinate. The total volume of the reaction mixture was 250 μl, and the final concentrations of DCPIP and sodium azide were 100 μM and 4 mM, respectively. Immediately after the addition of succinate, the plate was placed in a BioTek Synergy MX plate reader (BioTek, Winooski, VT, USA) pre-heated to 37°C, and reaction rates were measured for 3 min at 600 nm. Protein content of the mitochondrial preparations was measured with a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The reaction rates for each experiment were normalized by protein concentrations and expressed as ΔAU/min/mg protein.

Molecular Modeling We used molecular docking to predict the predominant binding mode(s) of PA with SDA. Successful docking methods search high-dimensional spaces of proteins and use a scoring function that ranks candidate dockings of compounds (i.e., ligands) to the protein. Docking can be used to virtually screen a large number of compounds, rank the results, and propose structural hypotheses that describe how the compounds interact with the target protein. A typical docking experiment involves computationally preparing the protein and the ligand, running the docking software, and evaluating the results. Advanced descriptions and specific methods used in our experiment are described in Supplementary Material 1.

In general, we prepared the mitochondrial respiratory membrane protein complex II (C-II) for molecular docking experiments by obtaining the crystal structure of the protein and using standard approaches to optimize the protein for docking. C-II contains four proteins, the flavoprotein containing the SDH enzyme domain (Chain A, SDHA), the iron-sulfur protein (Chain B, SDHB), the large (Chain C, SDHC; cytochrome b large, CybL) and small (Chain D, SDHD, CybS) trans-membrane proteins. Chains B, C, and D are relevant to binding of ubiquinone, a natural substrate for C-II.

The compounds 2-thenoyltrifluoroacetone (TTFA), ubiquinone-5 (UbQ), α-tocopheryl succinate (TOS), and PA were prepared for docking by using steps similar to those used to prepare the protein. The co-crystal structure coordinates of TTFA and UbQ (Sun et al., 2005) were chosen to validate docking experiments (Supplementary Material 1). In addition, the predicted UbQ binding free energy provides a natural substrate reference to compare to the binding strength of PA. The binding energy for TOS, a known inhibitor of the UbQ binding sites in C-II (Dong et al., 2008), was used in the docking experiment for comparison. Grid maps were created using AutoGrid version 4.2 (Morris et al., 2009) to describe interaction energies for each compound with SDH, one for each atom type in the compound. An affinity grid was created that centered on the protein ubiquinone binding site on C-II, specifically the proximal (Qp) and distal (Qd) sites.

AutoDock version 4.2 (Morris et al., 2009) with the Lamarckian genetic algorithm was used to simulate compound-SDH docking. TTFA was used as a positive control to optimize docking parameters to the Qp and Qd sites of C-II (PDB code 1ZP0). Conformational clustering was used to determine how often a given binding mode was predicted across all the dockings that were run. Docking results were obtained from the lowest binding energy conformations of the most populated cluster. The final docked structures were visualized by using visual molecular dynamics (VMD, Humphrey et al., 1996).

Statistical Analysis Reaction velocities and enzyme kinetics were determined using Microsoft Excel. V_{max} and K_m of the enzyme reaction were derived from Lineweaver-Burk plots. The inhibition constants (K_{iEIS}) were derived from the intercepts on axis of the secondary plots of $1/V_{max}$ vs. inhibitor concentration. The initial experiments indicated that the reaction rate reached maximum velocity with 5 mM succinate in the incubation mixture. Thus, 10 mM succinate was used to ensure enough substrate in the reaction to examine inhibition by PA at various concentrations (10–

160 μM). Percent inhibition by PA or MA was calculated for each inhibitor treatment. V_{max} (without inhibitor) or apparent $V_{max\ app}$ (with inhibitor), K_m (without inhibitor) or apparent $K_{m\ app}$ (with inhibitor), K_{cat}/K_m (catalytic efficiency), K_{iEIS}, and percent inhibition were compared among animal species within inhibitor treatments and within animal species among inhibitor treatments using a one-way analysis of variance (ANOVA). Tukey post-hoc tests were used for comparisons among multiple groups, and Dunnett post-hoc tests were used for comparison between treatments and control. The level of significance was $P<0.05$. Data for V_{max}, K_m and K_{iEIS} are presented as mean±standard error of the mean (SEM).

Results

Kinetics of SDH from Liver Tissues Excellent linearity was observed for progress curves during the 3 min period monitored. There was negligible reaction when succinate was omitted from the incubation mixture. The reaction rate increased with increases in succinate concentration and reached a maximum velocity at 5 mM succinate. Significant differences in V_{max} and K_m were observed among hares, mice, and rats (Table 1). The catalytic efficiencies (k_{cat}/K_m) for the enzyme from snowshoe hares and mice (18.6±1.5 and 15.9±0.9 min^{-1} mM^{-1}, respectively) were higher than the value for rats (13.5±0.6 min^{-1} mM^{-1}). Significant differences in $V_{max\ app}$ were observed among snowshoe hares, mice, and rats for all inhibitor treatments (Table 1). The $K_{m\ app}$ was significantly lower for enzyme from rats compared to enzyme from mice or snowshoe hares at low concentrations of PA, but the $K_{m\ app}$ did not differ when PA was 40 μM or higher or when MA was the inhibitor (Table 2). No difference was found between $K_{m\ app}$ for enzyme from snowshoe hares and mice for any inhibitor treatment.

Inhibition of SDH by PA All concentrations of PA (10–160 μM) inhibited SDH from all three animal species (Supplementary Material 2). PA appeared to act as an uncompetitive SDH inhibitor as indicated by the similar slopes in the Lineweaver-Burk plots (Supplementary Material 3). MA acted as a competitive inhibitor (Supplementary Material 3). Incubation with even very low concentrations of PA (10 μM) decreased $V_{max\ app}$ in a concentration-dependent manner in all animal species (Table 1). Similar patterns also were found for $K_{m\ app}$ (Table 2); K_m decreased with increasing concentration of PA, but $K_{m\ app}$ increased in the presence of MA.

The secondary plot of $1/V_{max}$ vs PA concentrations was linear for the enzyme from all animal species (Supplementary Material 4), confirming that PA is an uncompetitive inhibitor of SDH. The intercept on the X-axis of this plot indicates that K_{iEIS} for snowshoe hares (54.2±6.3 μM), mice (74.8±2.2 μM), and rats (106.7±1.4 μM) differ significantly ($P<0.05$; One-way ANOVA with Tukeys HSD test). SDH from snowshoe hares was more susceptible to PA inhibition (i.e., lower K_{iEIS}) compared to mice and rats (Supplementary Material 4).

The percent inhibition of SDH activity by PA was dose-dependent in all species (Fig. 1), with the enzyme from snowshoe hare most susceptible to inhibition. The inhibition of SDH by 80 μM MA was comparable to inhibition by 40 μM PA, but was significantly lower than inhibition by 80 μM PA, indicating that PA is a stronger SDH inhibitor than MA (Fig. 1).

Table 1 Maximal velocities of succinate dehydrogenase from liver mitochondria of snowshoe hares, C57BL/6 mice, or Wistar rats. Velocities (AU/mg protein/min) were determined in the presence ($V_{max\ app}$) or absence (V_{max}) of the inhibitors papyriferic acid (PA) or malonic acid (MA)

Inhibitor	Snowshoe hares	Mice	Rats
None	1.58±0.08[a]	1.85±0.01[b]	1.19±0.04[c]
PA, 10 μM	1.33±0.03*[,a]	1.58±0.02*[,b]	1.02±0.02*[,c]
PA, 40 μM	0.84±0.01*[,a]	1.07±0.04*[,b]	0.82±0.03*[,c]
PA, 80 μM	0.56±0.01*[,a]	0.84±0.02*[,b]	0.62±0.03*[,c]
PA, 160 μM	0.38±0.02*[,a]	0.56±0.01*[,b]	0.46±0.01*[,c]
MA, 80 μM	1.30±0.13*[,a]	1.75±0.20*[,b]	0.92±0.04*[,c]

Values represent mean±SEM for enzyme from four animals with and without PA and three animals for MA. An asterisk (*) represents a significant differences in means from no inhibitor within an individual species ($P<0.05$; *One-way ANOVA* with *Dunnett test*). Different letters indicate significant differences in means between species for an individual treatment ($P<0.05$; *Tukeys HSD test*).

Table 2 The Michaelis constant for succinate dehydrogenase from liver mitochondria of snowshoe hares, C57BL/6 mice, or Wistar rats. The K_m (mM) was determined in the presence ($K_{m\ app}$) or absence (K_m) of the inhibitors papyriferic acid (PA) or malonic acid (MA)

Inhibitor	Snowshoe hares	Mice	Rats
None	0.28±0.01[a]	0.29±0.01[a]	0.20±0.01[b]
PA, 10 μM	0.26±0.02[a]	0.25±0.01[a]	0.19±0.02[b]
PA, 40 μM	0.18±0.02* [a]	0.20±0.01* [a]	0.18±0.02[a]
PA, 80 μM	0.14±0.02* [a]	0.15±0.01* [a]	0.14±0.01* [a]
PA, 160 μM	0.07±0.01* [a]	0.09±0.01* [a]	0.08±0.01* [a]
MA, 80 μM	1.42±0.29* [a]	1.99±0.42* [a]	1.18±0.21* [a]

Values represent mean±SEM for enzyme from four animals with and without PA and three animals for MA. An asterisk (*) represents a significant differences in means from no inhibitior within an individual species ($P<0.05$; *One-way ANOVA* with *Dunnett test*). Different letters indicate significant differences in means between species for an individual treatment ($P<0.05$; *One-way ANOVA* with

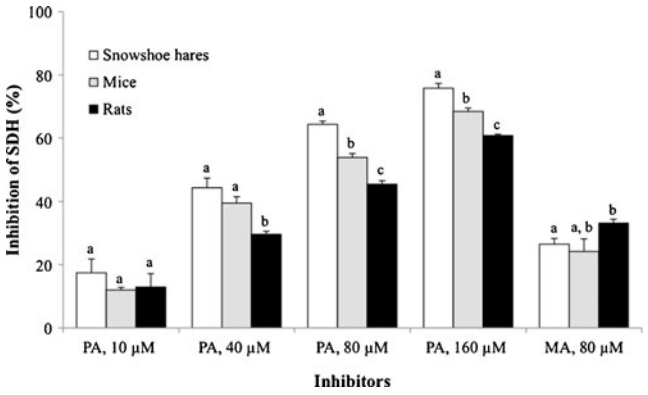


Fig. 1 Inhibition of SDH from snowshoe hares, C57BL/6 mice, and Wistar rats by papyriferic acid (PA) or malonic acid (MA). Inhibition is calculated as a percent of the activity of control samples from the same species. Values represent mean±SEM of 4 animals for PA and 3 for MA. Different letters indicate significant differences in means between species for an individual treatment ($P<0.05$; *Tukeys HSD test*)

Molecular Modeling When evaluating the results of docking, the main criterion to consider is how well the binding mode predicted by the docking matchs structural data, where available. TTFA was used as a positive control to show that AutoDock 4.2 is able to emulate experimental binding with an acceptable degree of accuracy and precision, and to validate further docking experiments with other inhibitors. Specifically, TTFA was re-docked to the proximal UbQ-binding site (Qp) and the distal UbQ-binding site (Qd) of C-II crystal structure (Fig. 2). The overall root mean square deviation of the predicted binding pose compared to the crystal structure pose is 2.67 Å for the Qp site and 2.81 Å for the Qd site. The differences between docked and crystal poses lie primarily in the placement and orientation of the thenoyl group, which is thought to interact with both binding sites through mediating water molecules. Additionally, UbQ was re-docked back to the Qp site. The docked quinone ring coordinates are in good agreement with those in the crystal structure (PDB code 1ZOY, Fig. 2).

Molecular modeling reveals that PA binds to both Qp and Qd sites in a fashion similar to TOS (Fig. 3). In the Qp site, the carbocyclic rings of PA reside in the same binding groove as the phenyl ring of TOS. The carboxylate groups of PA and TOS reside in the central binding pocket where the UbQ ring and the TTFA thenoyl ring are bound, making hydrogen bonds and electrostatic contacts with SER42 and ARG46, and interacting with TYR91 through bridging water. Similar to the isoprenoid tail of UbQ, the remaining hydrophobic side chain of TOS loops around the hydrophobic binding grooves adjacent to the central binding pocket. In contrast, PA adopts a horizontal configuration due to the favorable polar interactions between the carbocyclic ring ester and hydroxyl groups and ARG93, TRP173, and ASN174 residues.

In the Qd site, the carbocyclic rings of PA and the phenyl ring of TOS bind in the same location where the thenoyl ring of TTFA binds, at the center of the binding site. The succinate ester moieties of PA and TOS extend towards the bottom of the binding site. The end carboxylate groups of PA and TOS nearly overlap, indicating key electrostatic interactions and hydrogen bonding with positively charged LYS135 and LYS128 residues. Due to its large size, the carbocyclic rings of PA readily interact with TYR61 and TRP134 side chains without water mediation.

The predicted binding free energies indicate that PA has a higher affinity for both the Qp and Qd sites than TTFA, UbQ, or TOS (Table 3). Furthermore, UbQ, TOS, and PA have higher affinity for the Qd site than the Qp site, whereas TTFA seems to preferentially bind to the Qp site. These findings are corroborated with the crystal structure electron density data (Sun et al., 2005) and a molecular docking study using AutoDock 3 (Dong et al., 2008).

Discussion

We showed that inhibition of mitochondrial SDH is one mode of action of a secondary metabolite in birch that could deter feeding by snowshoe hares. Papyriferic acid, the major PSM of the juveniles of *B. neoalaskana* and *B. pendula*, inhibits SDH by an uncompetitive mechanism in a dose-dependent manner (Supplementary Material 2, 3, and 4). In addition, molecular modeling strongly supports the notion that inhibition of SDH activity is a result of PA targeting the ubiquinone binding sites rather than the catalytic succinate binding site in C-II (Fig. 3). PA and TOS bind to both ubiquinone sites in a similar fashion, which is distinct from TTFA and UbQ binding due to structural differences. However, the core binding regions are consistently occupied by all four ligands, indicating that TTFA, TOS and PA all disrupt ubiquinone binding. The predicted binding free energies generated by AutoDock show that PA is a significantly more potent ligand than TTFA and TOS at both sites (Table 3). Several factors may contribute to the higher potency of PA. First, PA has more polar ester, hydroxyl, and carboxylate groups than TTFA and TOS to enhance the hydrogen bond and electrostatic interactions with binding site polar and positively charged residues. Second, the large ring scaffold of PA increases potential π-π interactions and minimizes the need for water mediation. The *in vitro* and computer modeling results provide a mode of action for toxicity that may be responsible for the concentration-dependent anti-feedant effects of PA observed in snowshoe hares (Reichardt et al.,

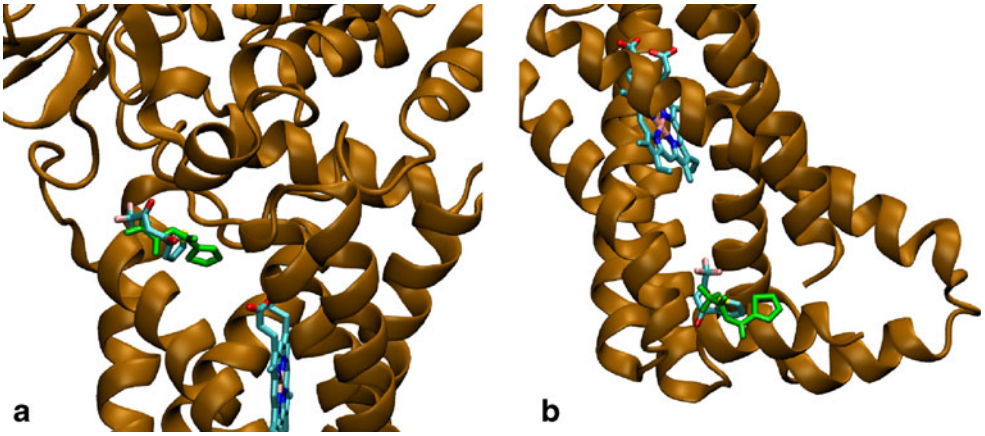


Fig. 2 Re-docking of 2-Thenoyltrifluoroacetone (TTFA) to the crystal structure of Complex II (C-II, PDB code 1ZP0). The predicted TTFA binding pose is colored by element and the crystal structure TTFA binding pose in green. The heme group is shown in cyan. (**a**) Qp site and (**b**) Qd site

1984). We predict that mechanisms that lower systemic concentrations of PA would reduce the toxicity of PA and explain the variable tolerance to PA observed between and within species that consume birch.

Inhibition of SDH may be a general mechanism of action of PA. PA inhibited SDH in a species that has had an ecological history with birch (snowshoe hares, this study) and those that are considered naïve to the chemicals in birch (ox, rabbits, rats, and mice, this study and in McLean et al. 2009). In every species tested thus far, inhibition of SDH by PA was dose dependent, uncompetitive, and more potent than a well-known competitive inhibitor of PA, malonic acid (Pardee and Potter, 1949; Greene and Greenamyre, 1995). From an evolutionary perspective, our *in vitro* results may at first appear somewhat puzzling. One might expect that animals with a 6000 year ecological history of consuming PA (Bryant et al., 2009), would have lower sensitivity to its MOA than naïve species. Lower sensitivity would provide a plausible explanation for the capacity of some hare populations to eat a PA-rich diet

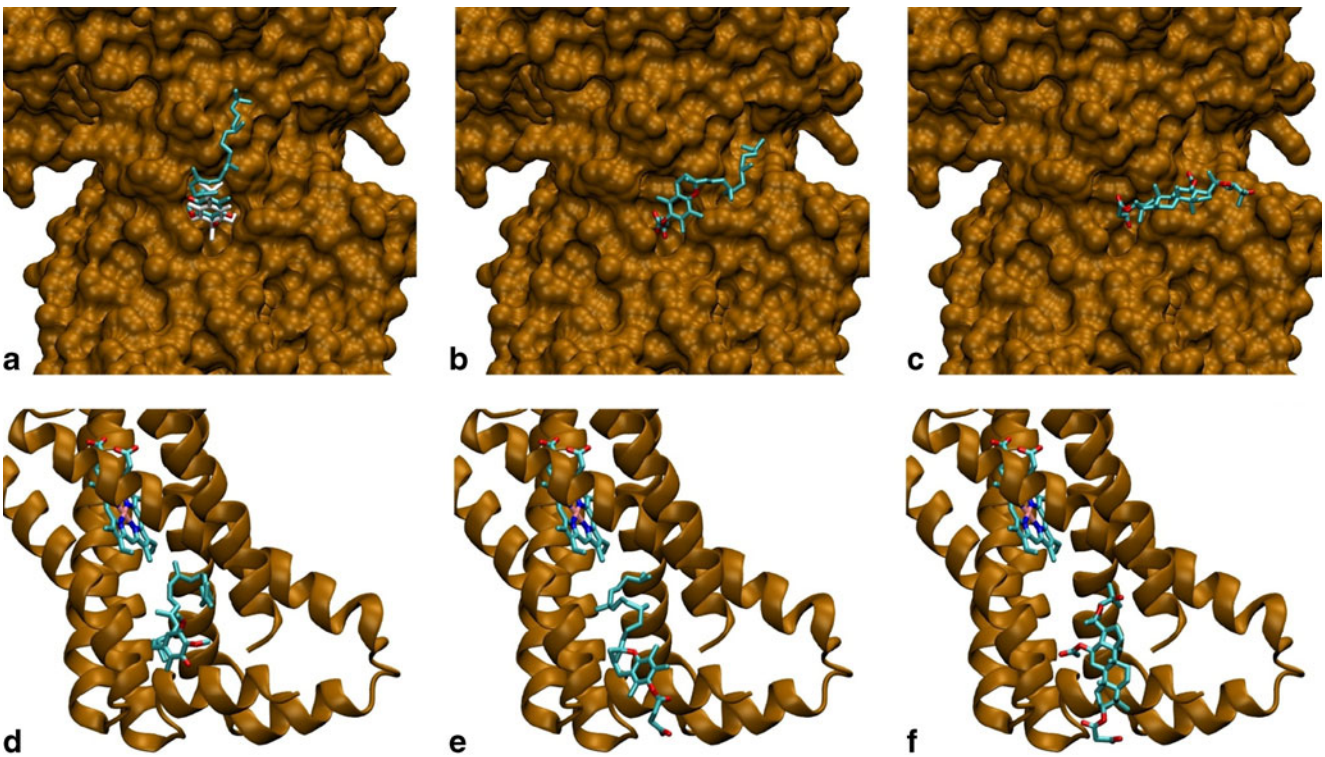


Fig. 3 Predicted binding poses of (**a**) Ubiquinone-5 (UbQ, cyan) and crystal structure UbQ (white), (**b**) α-tocopheryl succinate (TOS), and (**c**) papyriferic acid (PA) for the Qp site rendered in molecular surface representation, and (**d**), (**e**), (**f**) following the same order for the Qd site rendered in cartoon representation. The heme group is shown in cyan situated above the docked ligands

Table 3 Interaction energies (Kcal/mol) for the best-ranked docking conformations for 2-Thenoyltrifluoroacetone (TTFA), Ubiquinone-5 (UbQ), α-Tocopheryl succinate (TOS), and papyriferic acid (PA) in the Qp- and Qd-binding sites of succinate dehydrogenase. More negative interaction energies are indicative of higher binding affinity

Ligand	Qp site (Kcal/mol)	Qd site (Kcal/mol)
TTFA	−4.13	−3.99
UQ5	−5.15	−8.30
TOS	−6.55	−10.58
PA	−8.98	−12.55

(Bryant et al., 1994). In contrast, however, we have shown that snowshoe hare mitochondria are more sensitive *in vitro* than the naïve species tested.

In vitro sensitivity to PA inhibition may not translate to *in vivo* sensitivity for several reasons. First, the gut mitochondria of hares may be less sensitive to inhibition by PA than liver mitochondria. If absorption of PA by hares is low, as is the case in rats (McLean et al., 2009), then PA concentrations could be very low in the liver, and the gut would be a more plausible site of action than the liver. Moreover, the population of snowshoe hares we studied may not represent the species as a whole. Species do differ in their sensitivity to SDH inhibition (this study and Mogi et al., 2009), and we predict that the Alaska population of hares used here may have had higher sensitivity to SDH inhibition due to their "intermediate" tolerance to birch than would have been found in more tolerant populations (Swihart and Bryant, 2001; Bryant et al., 2009). Future studies could be performed to determine whether a geographical mosaic exists for sensitivity to SDH inhibition by PA.

An alternative explanation for our results is that the *in vitro* sensitivity to PA by hares does not account for *in vivo* mechanisms that can influence the systemic dose of PA that interacts with SDH in naturally feeding animals. The dose-dependent inhibition found in a variety of species suggest that variation in tolerance of PA by species or populations could be associated with physiological mechanisms that reduce systemic concentrations of PA rather than mechanisms that influence the sensitivity to inhibition of SDH by PA. Hares can behaviorally reduce the systemic dose of PA by avoiding the highest concentration of PA found in plant parts (Reichardt et al., 1984). In addition, enzymes that regulate absorption and metabolism of chemicals can dictate the concentration of ingested chemicals at the site of action and ultimately influence susceptibility to those chemicals (Danhof et al., 2007; Megarbane et al., 2008; Ploeger et al., 2009; Dahl et al., 2010). Efflux transporters and metabolizing enzymes could regulate the cellular concentrations of ingested PSMs in herbivores (Dearing et al., 2005; McLean and Duncan, 2006; Sorensen and Dearing, 2006; Sorensen et al., 2006; Forbey and Foley, 2009). Additional studies are needed to test whether efflux transporters and metabolizing enzymes can explain the disconnect between sensitivity to PA by snowshoe hares observed in these *in vitro* assays compared to the *in vivo* tolerance to PA by some populations of hares (Swihart and Bryant, 2001; Bryant et al., 2009).

This study is the first to our knowledge to confirm a mode of action of PA in an herbivore. Identifying the mode of action provides the necessary mechanistic data to help explain Thompson's (2005) geographic mosaic of selection in the birch-snowshoe hare system (Bryant et al., 1994, 2009; Swihart and Bryant, 2001). We have established a relatively simple protocol for testing SDH inhibition as a general mechanism of resistance in birch against other herbivores. SDH inhibition also may be a general mechanism of toxicity of other PSMs in other plant-herbivore systems. In addition, we have showcased the value of molecular modeling to further elucidate the mechanism of toxicity of PSMs and other ecologically relevant molecules. This approach has been fruitful in predicting binding of PSMs to metabolizing enzymes in insects (Baudry et al., 2003; Rupasinghe et al., 2003; Jones et al., 2010; Mao et al., 2007) and could prove equally valuable in screening the binding between PSMs and efflux transporters and metabolizing enzymes as well as the mode of action of PSMs in vertebrates.

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(Schwachtje and Baldwin, 2008), and will complement our extensive knowledge of herbivory's effect on plant secondary metabolites (Rosenthal and Berenbaum, 1992; Zhao et al., 2005; Pauwels et al., 2009).

Primary metabolic changes related to direct resistance have been investigated on a local scale. For example, leaf wounding can induce increased carbon sink strength through increased invertase activity, which drives influx of sucrose (Roitsch and González, 2004). This additional photosynthate can be allocated to defense-related processes, such as production of defensive enzymes (e.g., polyphenol oxidases in tomato, Bhonwong et al., 2009) or defensive phenolic small molecules via the shikimate pathway (Arnold and Schultz, 2002). For example, in a recent study, Hanik et al. (2010) studied induction of the aromatic amino acids derived from the shikimate pathway tobacco plants treated with methyl jasmonate (MeJA), a known defense elicitor (Koo and Howe, 2009; Pauwels et al., 2009). They observed rapid changes: 4 hr post-treatment, newly synthesized aromatic amino acids were increased, and at 15 hr tryptophan remained at a higher concentration relative to baseline levels. On the other hand, *trans*-cinnamic acid, an upstream compound in phenolic metabolism, did not increase until 15 hr post-treatment, suggesting a time lag between changes in primary and secondary metabolism. Despite findings such as this at the site of elicitation, primary metabolism's role in resistance is less well characterized at the whole-plant level. It is unknown, for example, how these metabolites change systemically or in response to different herbivores.

In addition to contributing to direct resistance, rapid changes in metabolite allocation may serve a role in tolerance to herbivory (Schwachtje et al., 2006). Specifically, induced reallocation can occur rapidly while the herbivore is still present, sequestering the primary metabolites from exposed tissues into storage organs (Orians et al., 2011). This process presumably functions to safeguard resources in herbivore-inaccessible tissues for use in future plant regrowth. Induced tolerance responses of this kind contrast with the traditional notion of constitutive tolerance, where resources are mobilized from storage organs to growing tissues only after the herbivory threat is over (Iwasa and Kubo, 1997; de Jong and van der Meijden, 2000).

Induced changes in resource allocation have been the focus of several recent studies that use radioisotopes to track, in living tissue, carbon/nitrogen uptake, transport, and allocation on the whole-plant level. In the pioneering study by Babst et al. (2005), [^{11}C] radiolabeled CO_2 was used to track how recently assimilated carbon was allocated within *Populus tremuloides* saplings after local application of MeJA. Hormone treatment led to increased speed of leaf carbon export and increased [^{11}C]-photosynthate allocation towards roots and stem (storage organs). A similar study in *Nicotiana attenuata* showed a 10% increase in [^{11}C]-photosynthate export from leaves to roots 4 hr after simulated herbivory (Schwachtje et al., 2006). More recently in tomato (*Solanum lycopersicum*), Gómez et al. (2010) showed that inducing herbivory responses through MeJA treatment increased both [^{11}C]-photosynthate and [^{13}N]-amino acids export from treated leaves and increased [^{13}N]-amino acids into the roots. While these studies suggest that changes in resource allocation may be a general response to damage, the change of specific metabolites in different tissues (e.g., stem, root, and apex) remains unexplored. In addition, most previous studies of primary metabolism have focused only on changes within or between leaves (Staswick, 1994; Zangerl and Berenbaum, 1998; Van Dam and Oomen, 2008; Schmidt et al., 2009), rather than co-occurring responses at a whole-plant level.

The type of whole-plant response also is expected to vary by the type of attacking herbivore. Plants and herbivores that share a co-evolutionary history are in a constant "arms race," and plants show unique responses to specific attackers that optimize defense responses (Agrawal, 2000; Rodriguez-Saona et al., 2010; Chung and Felton, 2011). Different combinations of resistance and tolerance tailored to combat specific herbivores thus should lead to distinct primary metabolic responses in the target plant. Caterpillars of the specialist *M. sexta*, for example, are well-adapted to feed on plants of the Solanaceae family and can detoxify alkaloids such as nicotine (Morris, 1984; Wink and Theile, 2002). Generalist herbivores on the other hand, feed on multiple plant families but have a lower ability to withstand toxins (Ballhorn et al., 2010). Since plants are attacked by multiple kinds of herbivores in nature (Strauss, 1991; Vos et al., 2001), induction of herbivore-specific responses may increase fitness and survival. For example, attacked *Nicotiana attenuata* plants reduce normal wound-induced levels of nicotine production when exposed specifically to *M. sexta*, against which this defense would be ineffective (Kahl et al., 2000). Thus, it is hypothesized that herbivore identity will be a crucial factor in the activation of an induced resistance or induced sequestration strategy (reviewed in Orians et al., 2011).

To compare primary metabolic responses to herbivory, mass spectrometry-based metabolic profiling allows for unbiased measurement of dozens of small primary metabolites and their changes in response to biotic stress (Hofmann et al., 2010). Here, we made use of such tools in order to complement previous carbon and nitrogen radioisotope studies. Our specific goals were: a) to elucidate how tomato plants reprogram primary metabolism at a whole-plant level by looking at different plant tissues (apex, undamaged leaflets of damaged leaves, stem, and roots); and b) whether two tomato attackers with contrasting host specialization (specialist *M. sexta* and generalist *H. zea*) alter plant metabolism

in ways consistent with either an induced tolerance- or induced resistance-like response. We predicted that metabolic changes would be specific to single tissues, and that changes in compounds related to resistance (e.g., phenylpropanoid precursors, Dixon and Paiva, 1995) and tolerance (e.g., storage sugars and N-transporting amino acids) would be herbivore specific.

Methods and Materials

Plant and Herbivore Material "Lady First II" tomato plants were obtained from Hazzard's Wholesale Seeds (Deford, MI, USA), germinated indoors under grow lights in SunGro Metro-mix soil for 15 d, and repotted into 50/50 sand/zeolite growth medium. Plants were grown at Tufts University in a greenhouse from September to October 2009 with no supplemental lighting. Plants were fertilized with 50 ml Hoagland's solution (1306 μM NO_3^-, 603 μM Ca^{2+}, 190 μM PO_4^{3-}, 993 μM K^+, 270 μM Mg^{2+}, 272 μM SO_4^{2-}, 2 μM Mn^{2+}, 0.0085 μM Zn^{2+}, 0.15 μM Cu^{2+}, 20 μM B, 1.44 μM Mo^{2+}, 40.5 μM Fe^{2+}) twice weekly until 20 d post-sowing, when the phosphate component was doubled. Plants were fertilized thereafter with 100 ml of 2x phosphate Hoagland's solution three times weekly until harvest. The group of harvested plants had an average height of 25.7 cm and averaged 7.3 fully emerged true leaves.

Manduca sexta caterpillars were obtained from Tufts University's Department of Biology. *Helicoverpa zea* caterpillars were obtained from Pennsylvania State University's Department of Entomology. Feeding cues of *M. sexta*, a specialist, are known to induce numerous changes in tomato and other Solanaceous plants (Kessler and Baldwin, 2002; Schwachtje et al., 2006; Howe and Jander, 2008). Feeding by *H. zea*, the generalist, is less well studied, but like *M. sexta*, salivary secretions released upon leaf consumption may elicit metabolic shifts (Peiffer and Felton, 2005).

While several studies have used oral secretions from *M. sexta* as a defense elicitor (e.g., Kessler and Baldwin, 2002), it is possible that external application of regurgitant exposes plants to far more elicitor than is actually released in oral secretions during caterpillar herbivory (Peiffer and Felton, 2009). In addition, diurnal and nocturnal patterns of feeding can differentially affect responses (Arimura et al., 2008). Therefore, in order to maintain comparable and realistic damage treatments, we exposed plants to caterpillars rather than to oral secretions.

Damage Treatments and Tissue Collection One individual *M. sexta* or *H. zea* caterpillar was introduced onto the terminal leaflets of three non-orthostichous leaves 60 d post-sowing. The three newest fully expanded (>50% final area) leaves were considered source leaves and exposed to herbivore damage. These leaves were chosen because it has been shown that damaging source leaves (as opposed to sink tissues) is necessary to ensure systemic defense expression in other systems (Gómez and Stuefer, 2006). Damaging multiple leaves ensured a systemic signal despite sectoriality (Orians et al., 2002). *Manduca sexta* usually forages across the whole plant, and late instars can defoliate an entire plant overnight. We thus confined third instars to terminal leaflets by using clip cages in order to preserve leaf tissue for analysis and to standardize damage by the two herbivores. Feeding cages were set up at 9 AM, and caterpillars were allowed to feed for 24 hr. A full day of exposure to herbivory was chosen in order to expose plants to both diurnal and nocturnal herbivory, as well as to allow time for metabolite levels controlled by circadian rhythms (e.g., sucrose vs. starch balance, Smith and Stitt, 2007) to respond to herbivory. This also corresponds with previous studies that have demonstrated that certain tomato defense responses peak at 24 hr (Orians et al., 2000).

As controls, mechanical damage was applied to a separate set of plants by performing a single scissor clip to 3 terminal leaflets three times over the 24-h exposure, with a final removal of 50% leaflet area to achieve an amount comparable to the area removed in the herbivore treatments. Empty clip cages were attached similarly to the 3 mechanically-damaged leaves. Undamaged control plants also received an empty clip cage, and were manually manipulated in the same manner as damage-exposed plants. Plants from all 4 treatments were assorted randomly for the damage exposure period. Both species of caterpillars removed a similar estimated leaf area (32% +/− 19.1 for *M. sexta,* 45.4% +/− 12.8 for *H. zea*), minimizing the effect of damage magnitude on metabolite concentrations.

Four tissue types were sampled 24 hr after initial herbivore exposure: apical leaves (excluding any developing flowers), undamaged leaflets of the 3 damaged leaves (hereafter referred to as "leaves"), stems from uppermost root to base of the first apex leaf, and roots (after washing off soil substrate). Tissues were harvested, immediately submerged in liquid nitrogen, and stored at −80°C until further processing.

Metabolomic Analysis Tissues were kept separated by plant and homogenized after harvesting, being kept on liquid nitrogen for the duration of the grinding process. Leaf and apex tissue was ground with a frozen mortar and pestle, while stem and root tissue were ground in a ball mill for 1 min with pre-chilled milling chambers filled with liquid nitrogen. Tissue extracts were collected as previously described after adding a ribitol internal standard (Lisec et al., 2006). Vacuum concentrated samples were stored at −80°C at Tufts University and shipped to the Max Planck Institute for Molecular Plant Physiology.

Samples were reconstituted, and small metabolites were measured and analyzed by using GC-MS as previously described (Lisec et al., 2006). Metabolite peaks were determined using TagFinder (Luedemann et al., 2008), and metabolite content was normalized to ribitol internal standard. Retention time index and mass spectral tags for individual compounds were compared to in-house library sets (Erban et al., 2007; Hummel et al., 2007), providing a list of positively identified compounds. Because signatures of specific metabolites are determined from raw GC-MS data, this method yields an unbiased list of previously characterized metabolites that are present in a given tissue sample. Peak areas for specific metabolites were compared to undamaged plants, yielding relative metabolite concentrations after each damage treatment (absolute concentrations were unmeasured). Compounds were separated by category (amino acids, organic acids, sugars, and other compounds), and further specific categories (nitrogen-transporting amino acids, abundant cellular sugars, and shikimate-derived amino acid products) were considered for their roles in resource transport and resistance.

Statistical Analysis Principal component analysis (PCA) was performed using JMP 7.0 (SAS Institute 2007). Metabolite peak areas from GC-MS were log transformed, and missing data were replaced with concentration values of 0 (Kaplan et al., 2004).

Two-way MANOVA was performed with JMP, with treatment, tissue, and treatment by tissue interactions as factors and overall PCs 1–3 as responses. One-way MANOVA was performed for tissue-specific analyses, with tissue-specific PCs 1–3 as responses and treatment as the sole effect. A Wilks' *lambda* test was used to determine statistical significance of each effect.

Ribitol-normalized metabolite content for individual metabolites was averaged for 5 to 6 plants per treatment and compared to mean content from undamaged control plants using Student's *t*-test.

Results

Fifty-six primary metabolites were measured in total across all tissues, identifying many differences in metabolite concentrations relative to undamaged plants. Altered compounds included free amino acids, organic acids, sugars, and other detectable metabolites (Fig. 1). Although not all 56 compounds were measurable in every tissue type, individual compound detectability never varied across replicates or damage treatments within a single tissue type (raw data not shown).

As expected, there were extensive differences in metabolite concentrations among tissue types. Data were highly clustered by tissue in principal component analysis (Fig. S1), with the top 3 principal components explaining 94.3% of the total variation. The significance of tissue type on total metabolite profile was highly significant in a two-way MANOVA on the top 3 principal component scores ($F=2622$, $P<0.001$), and a marginal effect of the tissue by treatment interaction was also measured ($F=1.33$, $P<0.13$).

Within the separate metabolic environments of each tissue type (apex, leaves, stem, and roots), MANOVA on principal components revealed that damage treatments also led to broadly different metabolic profiles (Fig. S2). The effect of damage treatment on PC 1–3 scores was significant in all four tissues ($F>2.0$, $P<0.05$). Patterns of differentiation were different for each tissue, with the stem, root, and apex showing the greatest PC differentiation when comparing damage treatments to undamaged control plants.

In accordance with results from PCA, some tissues exhibited a greater number of changes in individual metabolites than others (Fig. 2). Specifically, the number of changes was highest in the apex, where 28 metabolites significantly changed following one or more damage treatment (representing 58.3% of all measured metabolites in this tissue). Root tissue also was highly altered, with 25 metabolites (65.8%) showing induced changes. In contrast, both stems and leaves exhibited only 12 significantly altered compounds. The number of responses only induced by either type of herbivory (but not mechanical damage) also was highest in the apex (33.3%) and in the roots (28.9%).

Damage-induced changes in individual metabolite concentrations ranged in magnitude from a 3.33-fold decrease to a 4.58-fold increase relative to undamaged plants. In each individual tissue, *H. zea* induced as many or more individual metabolite changes than *M. sexta*—counting both increases and decreases (Fig. 2a). *Helicoverpa zea* herbivory also was the only type of damage to induce any concentration increases in the leaflets adjacent to focal damage. Other patterns were notable: *M. sexta* was the only treatment to induce any significant concentration increases in the roots, and mechanical damage induced many more concentration decreases in the roots than either herbivory treatment.

Metabolite concentrations changes tended to be specific to a single type of damage, and relatively few significant changes were induced by all three types of damage (see overlapping center areas in Fig. 2a). Consistent with patterns in PCA clustering, the apex showed the greatest similarity of response across damage treatments, but other tissues showed few or no shared changes in compound concentrations. Shared responses to both types of herbivory also were relatively rare across all four tissues (see *H. zea* and *M. sexta* overlapping areas in Fig. 2a).

Relative to mechanical damage, herbivory tended to increase concentrations of individual metabolites. When

		Relative Values											
		LEAF			APEX			STEM			ROOTS		
		MD	Ms	Hz	MD	Ms	Hz	MD	Ms	Hz	MD	Ms	Hz
	Sample Size	5	6	6	6	6	6	6	6	6	6	6	6
Amino Acids	Alanine	1.01	1.17	1.14	1.04	1.17	1.03	0.97	0.90	0.93			
	Alanine, beta				1.10	1.24	1.11	1.09	1.14	1.15	0.95	1.31	0.86
	Arginine							1.02	0.95	0.95			
	Asparagine							0.71	0.71	0.69			
	Aspartate	1.08	1.06	1.40	1.07	1.02	1.12	0.97	0.96	1.02	0.81	1.26	1.08
	GABA	0.78	1.14	1.16	1.10	1.32	1.09	0.83	0.99	1.14	0.45	0.94	0.68
	Glutamate	0.88	0.87	1.06	0.73	0.83	0.82	0.86	0.88	0.78	0.81	1.11	0.89
	Glutamine	1.00	1.08	1.17				1.03	1.47	1.13			
	Glycine	0.71	0.95	0.90	0.62	0.86	0.63	1.00	0.93	1.21			
	Isoleucine	0.94	0.91	0.90	1.17	1.18	1.51	1.02	1.08	1.13	0.80	1.09	1.01
	Lysine	0.77	0.85	1.00	1.11	1.26	1.38						
	Methionine	1.01	1.40	1.49				0.96	1.05	1.03			
	Ornithine				1.08	1.33	1.18				1.11	0.91	1.32
	Phenylalanine	0.93	0.78	0.68	0.61	0.58	0.42	0.87	0.92	0.78	0.70	0.93	0.80
	Proline	0.93	1.09	1.21	1.24	1.48	0.68	0.85	0.88	0.82	0.70	1.37	0.82
	Serine	0.68	0.89	0.85	0.84	0.94	0.65	0.98	0.92	1.03	0.74	0.89	0.93
	Threonine	0.91	0.89	0.88	0.88	0.94	0.89	0.98	1.07	1.06	0.75	1.03	0.93
	Tryptophan	0.82	0.93	0.96	1.52	1.85	4.48	1.09	1.71	1.56	0.67	0.88	0.94
	Tyramine	0.91	0.95	0.96	0.97	1.01	0.99	1.43	0.96	0.78	0.73	0.85	0.78
	Tyrosine	1.04	1.03	1.01	1.11	1.29	1.55	1.05	1.08	1.20	0.73	0.94	0.87
	Valine	0.95	0.86	0.88	1.21	1.19	1.33	1.05	1.15	1.20	0.79	1.11	1.00
Organic Acis	Aconitic acid							0.84	1.18	1.08	0.87	1.24	0.90
	Caffeic acid	0.78	1.60	1.19	0.81	0.92	0.83						
	Citric acid	1.00	1.01	1.26	0.99	1.12	1.25	0.97	0.97	0.93	0.79	1.03	0.88
	Dehydroascorbic acid	0.83	0.95	0.75	0.64	0.30	0.30	1.19	1.13	0.77			
	Fumaric acid	0.70	1.01	1.04	0.59	0.79	0.74	0.86	1.34	1.25	0.81	1.25	0.94
	Galacturonic acid/Glucuronic acid	0.94	1.22	1.43	0.96	0.92	0.86	1.05	1.02	0.93			
	Glutaric acid, 2-oxo-	0.70	0.70	0.99				1.03	1.06	1.20	0.81	0.92	0.79
	Glyceric acid	0.51	0.69	0.73	0.47	0.59	0.53						
	Malic acid	0.80	0.85	1.00	0.81	0.73	0.85	0.84	0.86	1.01	0.86	1.13	0.97
	Pyruvic acid	0.66	0.97	0.90	1.03	1.36	1.28	0.81	0.91	0.89	0.72	0.83	0.88
	Quinic acid	0.89	1.05	1.19	1.04	1.11	0.98	0.94	1.00	0.96	0.72	1.05	0.87
	Quinic acid, 3-caffeoyl-, cis-				1.13	0.99	1.22	0.90	1.60	1.30	0.80	1.36	1.27
	Quinic acid, 3-caffeoyl-, trans-	0.77	0.82	0.89	0.90	0.72	0.59	1.15	0.88	0.80	0.96	0.65	0.72
	Saccharic acid	1.04	1.21	1.55	1.21	1.32	1.37	1.07	1.09	1.19	0.64	1.53	1.08
	Succinic acid	0.91	0.92	1.16	0.64	0.77	0.78	1.02	1.02	1.11	0.82	0.98	0.84
	Threonic acid	0.95	1.04	1.22	1.11	0.91	1.15	0.97	0.97	1.03			
Sugars & Sugar Alcohols	**Fructose**	0.89	1.03	1.04	0.47	0.70	0.47	1.00	0.72	0.94	0.96	0.95	0.81
	Fructose-6-P	0.89	0.95	1.02				0.83	0.96	0.95			
	Fucose	0.91	0.93	0.99	0.99	1.05	0.87	1.01	1.02	0.95	0.78	1.00	0.78
	Galactinol	0.75	0.94	0.95	1.17	1.45	1.12	0.89	0.90	0.90	0.89	1.39	0.96
	Galactose	0.87	1.04	1.05	0.77	0.84	0.82				0.94	1.07	0.78
	Glucose	0.84	0.95	0.92	0.61	0.61	0.43	0.92	0.84	0.90	1.04	0.98	0.81
	Isomaltose	0.91	0.91	0.96	0.95	0.85	0.91						
	Maltose	0.97	1.32	1.55	1.06	1.06	1.51				0.74	0.86	0.65
	Mannose	0.92	0.98	1.05	0.89	0.91	0.68						
	Mannopyranoside, 1-O-methyl-	0.83	0.90	0.91	0.90	0.90	0.90	0.98	1.06	0.92			
	Raffinose	0.82	0.72	0.90	0.90	0.94	0.82	0.79	0.69	0.60	0.81	0.97	0.62
	Rhamnose	0.79	0.87	0.89	0.94	0.98	0.94	0.94	0.94	0.92	0.78	1.06	0.83
	Sucrose	0.78	0.93	0.93	0.80	0.87	0.87	0.85	0.81	0.77	0.79	0.95	0.85
	Trehalose-α,α'							0.84	0.85	0.78	0.92	1.26	0.81
	Xylose	0.90	0.93	1.12	0.82	0.92	0.69	0.98	0.89	0.87	0.84	1.00	0.80
Misc.	Inositol, myo	1.09	0.76	0.92	0.90	0.88	0.97	0.92	0.94	0.92	0.75	1.03	0.90
	Phosphoric acid	0.85	0.96	1.21	1.35	1.18	1.32	1.10	1.07	1.21			
	Putrescine	0.94	1.32	1.71	0.96	1.03	0.94	0.93	0.87	1.02	0.39	0.77	0.61
	Urea				1.19	1.04	1.13						

◀ **Fig. 1** Mean relative concentration values of metabolites relative to undamaged control means (normalized to 1.0). Statistically significant differences from undamaged plants (Student's *t*-test, $P<0.05$) are highlighted with a shaded box. Sample size (number of harvested plants) is given above each tissue-treatment combination. Undamaged control sample sizes were 5 plants for apex and leaf (undamaged leaflets of damaged focal leaf) and 6 plants for stems and roots. Treatment labels: "MD", mechanical damage, "Ms", *Manduca sexta* herbivory, "Hz", *Helicoverpa zea* herbivory. Nitrogen-transporting amino acids, abundant cellular sugars, amino acid products of the shikimate pathway, and components of phenylpropanoid metabolism are underlined and highlighted in bold

compared to mechanically damaged plants, 42 measurements of metabolites across all tissues showed increased concentration in either *M. sexta* or *H. zea*-damaged plants, as compared to just 7 showing decreases (Fig. 2b, see Fig. S3 for compound list and relative values). Differences relative to mechanical damage were especially pronounced in the undamaged leaflets, where *H. zea* induced many unique increases, including increases in quinic acid, GABA, and putrescine. Differences also were striking in the roots, where *M. sexta* caused many unique inductions of concentration increases, including increased concentrations of aspartate and phenylalanine relative to mechanical damage.

In addition to showing damage type-specificity, responses also were highly specific to single tissues. The most common damage response for a given metabolite was a change specific to 1 of the 4 tissues (Fig. 3); this held true for responses to all three damage types. Relative to mechanical damage, herbivory led to a larger number of ubiquitous responses for individual metabolites. For example, herbivory altered nearly twice as many metabolites in two or three tissue types simultaneously (Fig. 3). Nonetheless, no single damage treatment caused changes in a compound across all 4 tissues.

In accordance to the number of overall changes in apex and root tissues (Fig. 2a), metabolite changes unique to a single tissue tended to occur in tissues far from the site of damage, rather than in the leaves. For example, for the 30 metabolites measurable in all 4 tissues, herbivore-induced change was most often exclusive to the apex: both *M. sexta* and *H. zea* herbivory induced apex-specific changes in 8 such compounds. For metabolites showing changes in more than one tissue (Fig. 3), simultaneous change in the root and apex was the most common combination of altered tissues: 5 of the 30 compounds showed changes in both tissues after *M. sexta* herbivory, and 3 compounds after *H. zea* herbivory.

To analyze these patterns in relation to tolerance and resistance, we examined specific changes in transport amino acids, sugars, and defense precursors. The levels of four major nitrogen-transporting amino acids—glutamate, glutamine, aspartate, and asparagine—were often altered relative

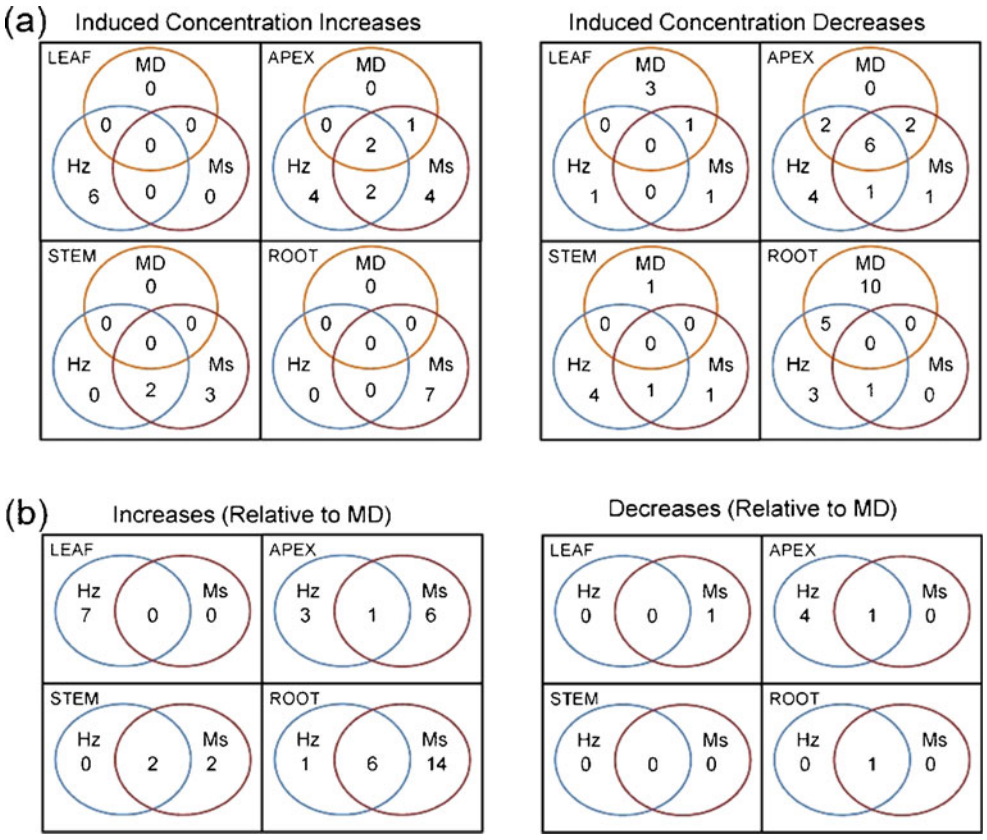


Fig. 2 Analyses of significant changes in individual metabolites relative to undamaged plants (Student's *t*-test, $P<0.05$) across tissues and damage treatments. **a** Venn diagrams of the number of metabolite concentrations that were significantly increased (*left*) or decreased (*right*). The total number of measured metabolites was 48 for leaves, 47 for stems, 48 for apex, and 38 for roots (see Fig. 1 for further information). Treatment categorizations: "MD", mechanical damage, "Ms", *Manduca sexta* herbivory, "Hz", *Helicoverpa zea* herbivory. **b** Venn diagrams of the number of altered metabolites after *M. sexta* or *H. zea* herbivory relative to mechanical damage

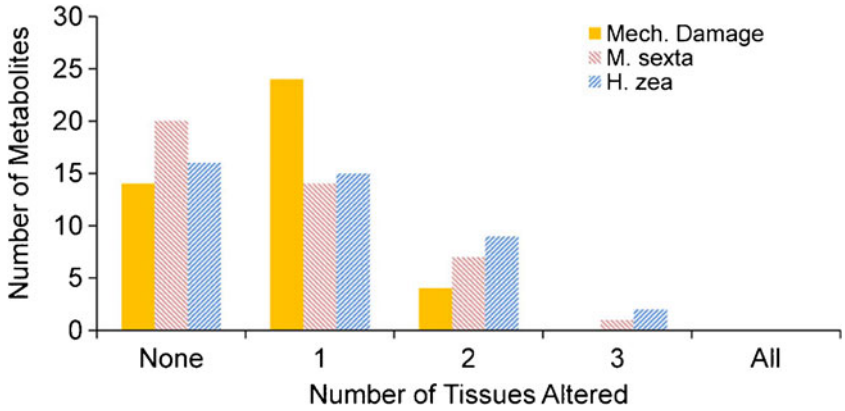


Fig. 3 Tissue-specificity of responses to damage. Histogram of the number of individual metabolites showing alteration after a specific damage type in 0, 1, 2, 3, or all tissues. Only the 42 metabolites measurable in 3 or more tissues were analyzed

to undamaged plants, depending on both tissue type and damage treatment (compound names in bold in Fig. 1). In the stem, glutamine levels increased in caterpillar-damaged plants, but only significantly so after *M. sexta* damage. In the leaf, *H. zea* induced more changes, causing significantly increased levels of aspartate. On the other hand, transport amino acid levels tended to decrease in the apex and root after damage. The apex contained detectable levels of only glutamate and aspartate, but glutamate was nearly significantly decreased after all three types of damage ($P<0.06$). Roots also showed decreases in glutamate and aspartate in response to mechanical damage, but slight increases after *M. sexta* herbivory.

Sugar concentrations, including those of the abundant cellular sugars glucose, fructose, and sucrose, tended to decrease after damage in all tissue types (Fig. 1). Decreases tended to be greater in tissues other than the leaf; herbivory led to only one significant change in any of the cellular sugars in leaf tissue (Fig. 1). Apex sugars in particular often showed significantly lower concentrations after all damage treatments. As with many metabolites measured, however, specific sugars often were altered only after a single type or damage or in a single tissue type.

Herbivory also induced changes in several small secondary metabolites and their immediate metabolic precursors. Changes in the aromatic amino acid products of the shikimate pathway—phenylalanine, tryptophan, and tyrosine—are highlighted in Fig. 1. Effects differed across tissues, but phenylalanine levels tended to decrease after herbivory, while tryptophan levels tended to increase (except in the roots). *Helicoverpa zea* induced the broadest changes in these metabolites, causing a significant decrease of phenylalanine in the leaf and apex, and mirroring increases of tryptophan and tyrosine in the apex. Damage also induced changes in metabolites downstream of the aromatic amino acids—precursors in the phenylpropanoid pathway (Fig. 1). Caffeic acid, quinic acids, and isomers of chlorogenic acid (*cis*- and *trans*- 3-caffeoyl-quinic acid) all showed unique local and systemic changes, often unique to a single herbivore.

Discussion

Herbivory induces primary metabolic responses in plants (Schwachtje and Baldwin, 2008), but the extent and type of these changes across different tissues or by different herbivores is poorly understood. Previous radioisotope work has demonstrated carbon and nitrogen reallocation after herbivory (Gómez et al., 2010) and induction of the shikimate/phenylpropanoid pathway (Hanik et al., 2010), but the specific primary metabolic changes involved in these shifts are less clear. We quantified primary metabolic changes following damage by two herbivores in the apex, undamaged leaflets of damaged leaves, stem, and roots of tomato plants. We observed whole-plant metabolic reconfiguration, with all four tissues showing global changes, as well as significant damage-induced changes in individual metabolites related to resistance and tolerance.

Interestingly, the sink tissues of the root and apex were more heavily altered (in terms of number and magnitude of changes) than tissues more proximal to damage—the leaves and stem. This is consistent with evidence that systemic signals are disseminated rapidly to distal leaves following wounding (Wasternack et al., 2006; Koo and Howe, 2009), and that whole-plant responses can lead to large-scale metabolic reprogramming after 24 h of damage. Despite an overall research focus on responses in the attacked leaf tissue (Kessler and Baldwin, 2002), this finding broadly justifies increasing interest in systemic, whole-plant responses to herbivory.

Responses in the apex and root were not only prevalent, but also showed different patterns unique to either *M. sexta* or *H. zea*. This is consistent with herbivore-specific elicitation of defense responses observed in other systems, relative to both undamaged and mechanically damaged plants (Agrawal, 2000; Kahl et al., 2000; Halitschke et al., 2001; Kessler and Baldwin, 2002). This result also suggests that distinct cues in the leaf are able to trigger dramatically different whole-plant responses, in agreement with recent studies of herbivore-specific salivary components and their different hormonal effects in the locally attacked leaf

(Peiffer and Felton, 2005; Diezel et al., 2009). For example, exposure of *Nicotiana attenuata* to regurgitant of *M. sexta* and *Spodoptera exigua* leads to distinct hormonal responses that lead to differential induction of nicotine and other defenses (Kahl et al., 2000; Winz and Baldwin, 2001; Diezel et al., 2009).

Surprisingly, we found that *H. zea* herbivory induced a wider metabolic reprogramming than other damage treatments in tomato. This contrasts with previous work on generalist-induced defense hormones and downstream defense responses: *S. exigua* regurgitant can antagonize anti-herbivore defenses (Diezel et al., 2009), and other studies have demonstrated defense silencing by generalist herbivores across various plant systems (Musser et al., 2002; Zarate et al., 2007). In our study, *H. zea* induced an equal or greater number of metabolic changes in each tissue relative to *M. sexta*. Changes in shikimate pathway products and simple phenylpropanoids, possibly characteristic of defense induction, also were stronger after *H. zea* herbivory, especially in the apex (see below). The heightened overall response to *H. zea* herbivory suggests that defense silencing by generalist herbivores may not always extend to the level of primary metabolism or whole-plant responses.

Previous studies have shown that herbivore attack leads to reallocation of carbon and nitrogen from the damaged leaves into storage tissues (Staswick, 1994; Beardmore et al., 2000; Babst et al., 2005, 2008; Schwachtje et al., 2006), often in a rapid manner known as induced sequestration (Orians et al., 2011). In tomato specifically, export of nitrogen from leaves into roots has been shown in response to MeJA (Gómez et al., 2010). We thus expected to see damage-induced differences in concentrations of nitrogen and carbon-transporting compounds relative to undamaged plants, especially after herbivory by *M. sexta.*

While whole-plant differences in individual sugars were apparent in response to both herbivores, data for the set of all measured sugars were relatively noisy. There was no consistent increase or decrease in any sugar, for example, in any one tissue in response to either herbivore. This suggests that the broad allocation changes measured by radioisotope analyses may occur at the level of storage compounds, in pools of starch or more complex carbohydrates, rather than in free or transporting forms of sugar represented by the primary metabolites measured by GC-MS. The fact that increased sucrose concentrations were not observed in any tissues, including the stem, also may indicate that translocation of carbon may already have occurred 24 hr after herbivory.

Regarding nitrogen, several whole-plant patterns were apparent in concentrations of the nitrogen-transporting amino acids—glutamine, glutamate, asparagine, and aspartate. While these amino acids did not show large changes in magnitude in response to any type of damage, *M. sexta* herbivory led to a significantly higher concentration of glutamine in the stem, as well as increased levels of aspartate in the root relative to mechanically damaged plants (Fig. 1, Fig. S2). While these changes did not hold for the other N-transporting amino acids, the lack of similar induced changes after *H. zea* herbivory may indicate a *M. sexta*-specific sequestration response. Future experiments that compare metabolite concentrations at different timepoints, along with measurements of storage compounds will help to resolve how herbivores differentially alter patterns of allocation and storage.

In addition to tolerance responses, production of direct defensive compounds is induced after herbivory (Rosenthal and Berenbaum, 1992; Karban and Baldwin, 1997). In tomato, phenolic compounds may play a role in anti-herbivore defenses (Dixon and Paiva, 1995), although the negative effects of specific compounds can be specific to only certain herbivores (Isman and Duffey, 1982; Bi et al., 1997). In the present experiment, *H. zea* induced greater changes in precursor amino acids of the shikimate pathway and simple phenylpropanoids than either *M. sexta* or mechanical damage. Shikimate-derived amino acids and simple phenylpropanoids are precursors for many secondary metabolites (Herrmann and Weaver, 1999), suggesting potential ecological consequences. Assaying additional defensive compounds as well as correlating metabolic changes with *H. zea* biomass gain on tomato could help to confirm a role for these primary metabolic changes.

While further speculation on the fate of carbon and nitrogen is possible based on the present data, more experiments are needed to determine the steps in reallocation of sequestered C and N. First, directly measuring phenolics, starch, protein, and other components of carbon and nitrogen allocation may reveal differences that account for the leaf export and root import of these resources (Gómez et al., 2010). Second, measuring metabolic flux of specific compounds will shed light on the pathways taken to achieve resource reallocation. Interpreting changes in metabolite concentrations along a pathway is complex, as concentration changes do not necessarily reflect turnover through the pathway (Last et al., 2007). Future experiments that measure a timecourse of metabolite concentrations following herbivory are needed to distinguish between general pathway downregulation or increased flux in the conversion of a metabolite to its products. Despite these limitations, the observed single timepoint changes in pathway components relative to undamaged plants – both increased and decreased pool sizes—are enough to suggest a differential prioritization of defense and resource allocation across the whole plant following contrasting damage types.

In summary, we found that primary metabolic responses across the entire tomato plant varied widely from tissue to tissue, were often herbivore-specific, and occurred in

metabolites potentially important to the response of tomato to different agents of damage. Looking at number and magnitude of induced concentration changes, tissues outside of the attacked leaf were most often affected by both *M. sexta* and *H. zea* herbivory, in patterns partially consistent with herbivore-specific defense and tolerance responses. Future findings on the effects of specific metabolites and biological implications of whole-plant change will increase our understanding of how plants defend against herbivory.

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al., 2011). To date, *de novo* oviposition-induced volatiles have been reported for some tritrophic systems. In some systems, the combination of oviposition and herbivory trigger volatiles that attract egg parasitoids (Colazza et al., 2004; Conti et al., 2010; Michereff et al., 2011).

Apart from oviposition-induced plant volatiles, the orientation of egg parasitoids towards egg-derived odors might be an effective alternative strategy for host location. However, eggs usually emit only small amounts of volatiles and are therefore only useful as short-range cues. Moreover, eggs generally are inconspicuous and deposited on hidden sites, making their location difficult (Vinson, 1998). This is why egg parasitoids are expected to frequently exploit chemical cues from other sources for host location as well. In the case of lepidopteran hosts, this includes, for example, pheromones and compounds released from scales of the wings of ovipositing females (Shu and Jones, 1989; Colazza et al., 1997), but also HIPVs (Lou et al., 2005; Moraes et al., 2005). Although HIPVs do not directly indicate the presence of eggs, their exploitation could be of use if the host has overlapping generations, as the highly abundant and easily detectable plant odors enable them to locate host habitats and communities, and, consequently, eggs (Vinson, 1976, 1984). Furthermore, HIPVs may help phoretic egg parasitoids to locate adults on which they can "catch a ride" to new oviposition sites with eggs (Fatouros et al., 2008).

Despite the possible importance of HIPVs for host location by egg parasitoids, few studies have directly addressed this subject (Reddy et al., 2002; Lou et al., 2005; Manrique et al., 2005; Moraes et al., 2005). Also, little is known about the capacity of egg parasitoids to learn HIPVs for host location (Mumm et al., 2005; Schroder et al., 2008). This is surprising, given the fact that learning is an important strategy for parasitoids that optimizes their host searching strategy (Lewis and Takasu, 1990; Turlings et al., 1993; Vet et al., 1995). To obtain deeper insight into these topics, we tested innate and learned responses of two lepidopteran egg parasitoids towards HIPVs that are released by maize plants upon attack by larvae of the noctuid moth *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae). We assessed the behavior of the generalist *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae), which parasitizes a wide range of hosts, (Zucchi and Monteiro, 1997), and the specialist *Telenomus remus* Nixon (Hymenoptera: Scelionidae), whose host range is limited to the genus *Spodoptera*. To complement this study, we also tested whether or not *S. frugiperda* gravid females show a preference for oviposition on plants that are damaged by conspecifics. We hypothesized that if moths prefer plants that have been damaged by conspecifics, or at least do not actively avoid them, HIPVs may guide egg parasitoids not only to habitats with hosts, but potentially also to plants containing host eggs of later arriving *Spodoptera* females.

Methods and Materials

Plants and Insects Maize seeds (*Zea mays* L., var. Delprim) were planted individually in plastic pots containing about 200 g potting soil ("Hortaliças Basiplant" substrate). Plants were grown in a greenhouse (12/12 h day/night cycle). Maize plants used in the bioassays had been growing for 8–9 d after emergence and had three fully expanded leaves. *Spodoptera frugiperda* caterpillars were collected on maize fields at Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba-SP, Brazil, and reared under laboratory conditions (25±3°C, 60±10% rh, 12/12 h). The caterpillars were reared individually in sterilized glass vials (8.5 cm high, 2.2 cm diam, plugged with cotton) on artificial diet (Greene et al., 1976). Emerging pupae were separated by sex and placed into PVC cages (10.0 cm diam and 22.0 cm high) that were closed at both ends with Petri dishes (14.2 cm diam). The Petri dish in the bottom of the cage was lined with filter paper. In addition, cages were lined with office paper to serve as a surface for oviposition. Adults were fed a 10% honey solution that was renewed every 2 d.

Both *Telenomus remus* Nixon (Hymenoptera: Scelionidae) and *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) were reared on 24 h-old *S. frugiperda* egg masses, which were placed in sterilized glass vials (9.0 cm high, 3.0 cm diam, sealed with PVC film) together with the parasitoids. One paper card containing eggs was offered per day in each glass vial. Each card was removed after a 5 h period and placed in a new glass vial. Upon emergence of the parasitoids, small droplets of honey were placed on the internal wall of the vials as food source. All vials were maintained in incubators (25±1°C, 60±10% rh, 12/12 h).

Moth Oviposition Preference In an oviposition preference test, we offered *S. frugiperda* females an undamaged maize plant (treatment UP) next to a caterpillar damaged plant (CD). To obtain CD, 10 sec-instar larvae were placed inside the whorl of the plants at 10:00am until the beginning of preference tests at 6:00pm. Plants of each treatment were placed on the opposite ends of a *voil* fabric cage (55.0 cm high, 60.0 cm long, 10.0 cm wide). At 6:00 pm, five 3-d-old gravid females were released in the cage and left for one night. The following morning, egg masses were removed from the plants, and the eggs were counted. The bioassay was replicated 20 times, and was conducted in a room under controlled conditions (25±1°C, 60±10 rh, 12/12 h).

Olfactometer Bioassays Olfactory responses of the egg parasitoids (*T. remus* and *Tr. pretiosum*) were tested for 3-to-4-d-old individuals that were either naïve or experienced. A Y-tube olfactometer was used to determine their preference, either between two different odor sources or one source and a blank (clean air). Experience was given to parasitoids by allowing

them to oviposit on 100 viable *S. frugiperda* while exposing them to HIPVs released by regurgitant treated (0–1, 2–3, 6–7, and 12–13 h) or to caterpillar damaged plants for 10 min. After this period, we collected only wasps that were parasitizing the egg masses. Wasps that were walking around the vial were not used as experienced wasps. Experienced parasitoids were tested on the subsequent day to the odors of the same treatment they had experienced against the undamaged plant. All bioassays were conducted in the laboratory (conditions as described above) during day time, between 10:00 and 17:00. The Y-tube olfactometer consisted of a bifurcated glass tube (2.5 cm diam), and two arms (9.0 cm long). The odor sources were placed inside glass bottles (11.5 cm i.d., 51 cm high), which were connected to the extremities of the olfactometer. From the main arm of the olfactometer, a tube from a vacuum pump was connected, and the air from the environment was purified with the use of granular activated carbon filter (40 mesh, 11 cm i.d., 35 cm high) before pulling it through the system. The air flow was adjusted to 300 ml/min using calibrated flowmeters connected to each arm. Insects then were positioned individually at the beginning of the central arm of the Y-tube and observed for 5 min. When the wasps crossed the threshold line (located in the middle of each arm) and stayed in the arm end for 20 sec, this was considered as "choice". Only insects that made a choice for one arm within the 5 min were considered for statistical analysis. Each parasitoid was used only a single time to prevent associative learning. After each trial, the olfactometer was disassembled and all glassware was washed with neutral dishwashing soap (v/v 5%), distilled water, acetone, and alcohol (v/v 90%).

To evaluate egg parasitoid responses to HIPVs, bioassays with the following combinations were carried out: (i) undamaged plant (UP) vs. blank (B); (ii) UP vs. regurgitant-treated plant (RT) at intervals 0–1, 2–3, 6–7, and 12–13 h after treatment; (iii) UP vs. caterpillar-damaged plant (CD), which was obtained by having 20 2nd instars feed overnight. The larvae were left feeding on the plant during the entire experiment. The treatment CD thus corresponds to a "host-plant-complex". The regurgitant was manually collected from 3rd-4th instar fall armyworm caterpillars fed on maize for 2 d and stored at −76°C. To simulate herbivory on plants, we scratched the 2nd and 3rd leaf (about 1 cm^2 of each) and applied 10 μl of regurgitant to each damaged site. Regurgitant-treated plants 0–1, 2–3, and 6–7 h were induced during the morning (8:00–10:00), and 12–13 h was induced at 22:00 and tested at 10:00 on the following day. For each bioassay, we conducted at least 30 replicates on 4–5 experimental days using different odor sources.

Volatile Collection and Analysis To analyze the volatiles released by regurgitant-treated (RT) and caterpillar-damaged plants (CD), maize seedlings were grown as described in Erb et al. (2010). Maize seedlings were subjected to *S. frugiperda* feeding and regurgitant treatment as described above and following the same timing as the bioassays. Regurgitant-treated plants (RT) then were placed into glass bottles (9.5 cm i.d., 54 cm high), and volatiles were sampled at intervals 0–1, 2–3, 6–7, and 12–13 h after induction using super-Q® filters. Caterpillar damaged plants (CD) were sampled on the following morning (10:00 am) after one-night of feeding, as wasps were tested at this time for attraction to CD odors. The super-Q® traps were extracted with 150 μl dichloromethane (Suprasolv; Merck, Dietikon, Switzerland), and 200 ng of n-octane and n-nonyl acetate (Sigma, Buchs, Switzerland) in 10 μl dichloromethane were added to the samples as internal standards. All extracts were stored at −76°C until analyses. Traps were washed with 3 ml dichloromethane before they were reused for a next collection. HIPVs of the experiments were identified with a gas chromatograph (Agilent 6890 Series GC system G1530A) coupled to a mass spectrometer that operated in electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33–280 amu). A 2-μl aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 μm film thickness; Alltech Associates). Helium at constant flow (0.9 ml/min) was used as carrier gas. After injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C/min followed by a postrun of 5 min at 250°C. Detected volatiles were identified by comparison of their mass spectra with those of the NIST 05 library and with the retention times of analyses from previous studies (Turlings et al., 1998; D'Alessandro and Turlings, 2006).

Response of Wasps to Synthetic Volatiles We measured the response of inexperienced *Tr. pretiosum* wasps towards different HIPVs in a Y-tube olfactometer following the procedure described above. First a synthetic GLV mixture comprised of 80% (*Z*)-3-hexanal, 10% (*Z*)-3-hexenol, 8% (*E*)-2-hexenal, and 2% (*Z*)-3-hexenyl-acetate (Sigma Aldrich, Switzerland) (Von Mérey et al., 2011), which corresponds to a typical GLV mix released by freshly wounded plants, was tested. Second, we evaluated the attractiveness of the sesquiterpene (*E*)-*β*-farnesene (Sigma Aldrich, Switzerland), a major sesquiterpene that is released from herbivore-induced maize plants. Hexane solutions of synthetic plant volatiles were prepared at concentrations of 1,000, 500, 50, and 5 μl/ml. For each replicate, 2 μl of synthetic plant volatile solution and hexane (control) were incorporated into filter paper squares of 2 cm^2, which were placed on the ends of olfactometer arms. Maize plants can release 2–3 μg of the tested volatiles following the first 12 h of induction (Erb et al., 2011), which means that the lowest concentration we tested can be reached by a stand of five or more infested plants in a dense canopy with little air circulation.

Statistical Analysis Levene's and a Kolmorogov-Smirnov tests were carried out to determine heteroscedasticity of error variance and normality of the data. Number of eggs from preference tests performed with fall armyworm moths were analyzed by paired *t-tests* (α=0.05). Differences between behavioral responses of parasitoids under pairs of treatments were analyzed by *chi-square* tests (α=0.05 and α=0.01). Individuals that did not make a choice were excluded from the statistical analysis. Statistical analysis was performed using the software Statistica 6.0.

Results

Herbivore Oviposition Choice. Spodoptera frugiperda females laid, on average, 283 eggs on undamaged plants (UP) and 293 eggs on caterpillar-damaged plants (CD). There was no significant difference between the treatments in number of eggs (paired *t* test, N=20, t=−0.0951, P=0.925) or number of egg masses laid by *S. frugiperda* (data not shown).

Parasitoid Response to Maize Volatiles Neither wasps *T. remus* nor *Tr. Pretiosum* distinguished the odor of undamaged plants (UP) and air from blank arms (data not shown). Therefore, for the following bioassays, UP was used as a control. Results in detail were as follows: Naïve *T. remus* females did not show any preference for odors emitted by regurgitant-treated plants (RT) 0–1 h (Fig. 1; Table 1). Experienced females preferred odors from RT 0–1 h to UP (Table 1). The generalist *Tr. pretiosum* showed a significant preference for RT 0–1 h odors independently of previous experience (Fig. 1; Table 1). For the following time point, again, naïve *T. remus* showed no preference for odors emitted by RT 2–3 h (Fig. 1; Table 1), but a significant number of experienced females preferred RT 2–3 h to UP (Table 1). Both naïve and experienced *Tr. pretiosum* females showed a significant preference for RT 2–3 h odors compared to UP (Fig. 1; Table 1). Both *T. remus* and *Tr. pretiosum* showed a similar response towards volatiles released from RT 6–7 h. Naïve wasps showed no preference to RT 6–7 h or UP (Fig. 1; Table 1), while, after oviposition experience, they significantly preferred the RT 6–7 h odors to UP (Table 1). Neither naïve nor experienced *T. remus* and *Tr. pretiosum* showed a preference to RT 12–13 h compared to UP odors (Fig. 1; Table 1). *Telenomus remus* and *Tr. pretiosum* females did not distinguish between the odors of caterpillar-damaged plants (CD) or UP (Fig. 2; Table 1). However, significantly more experienced *T. remus* wasps preferred CD odors to UP (Table 1). *Trichogramma pretiosum* experienced wasps did not distinguish between CD and UP odors (Table 1).

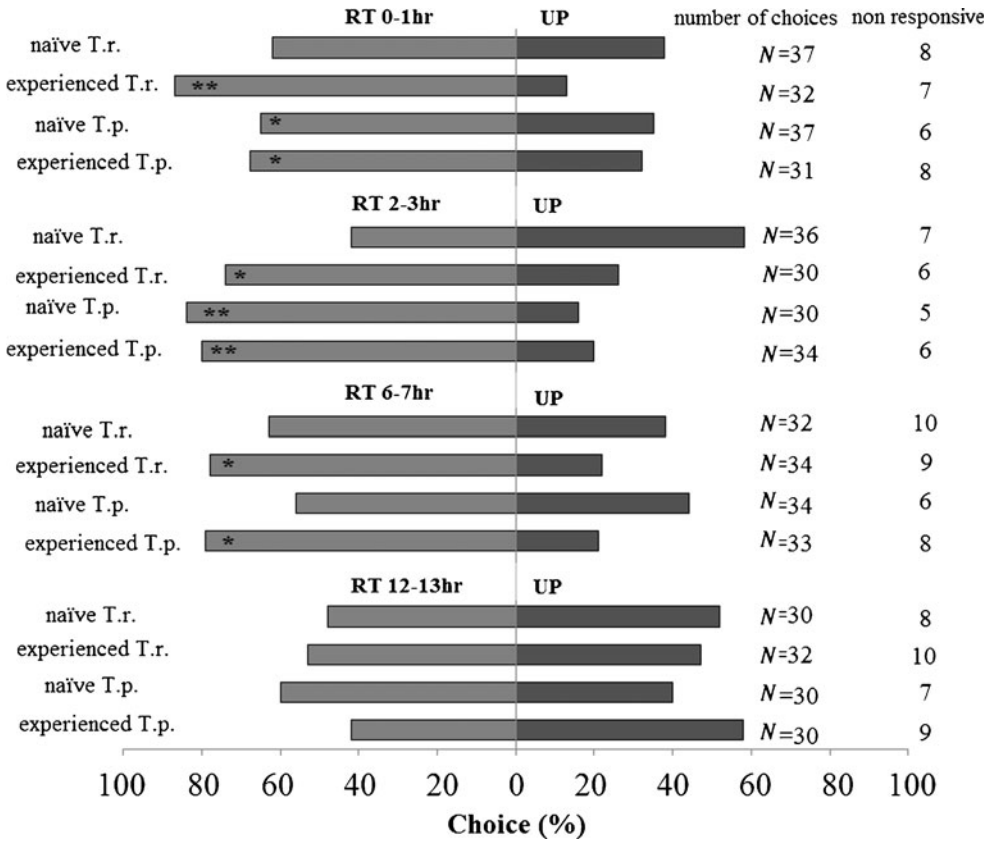


Fig. 1 Response of naïve and experienced *Telenomus remus* and *Trichogramma pretiosum* to volatiles emitted by undamaged plant (UP) and *Spodoptera frugiperda*-regurgitant treated plant (RT) 0–1, 2–3, 6–7, and 12–13 h. T.r. = *T. remus*; T.p. = *Tr. pretiosum*. * significant at 5% according to chi-square; ** significant at 1% according to *chi-square*

Table 1 Results of *chi-square* tests on the wasp choices in the Y-tube olfactometer between the undamaged plant and *Spodoptera frugiperda* regurgitant-treated (RT) or caterpillar-damaged plant (CD)

Wasp		Treatment	*N*	χ2	*P*
T. remus	Naïve	RT 0–1 h	37	2.189	0.139
		RT 2–3 h	36	1.000	0.317
		RT 6–7 h	32	2.000	0.157
		RT 12–13 h	30	0.133	0.715
		CD	39	0.100	0.753
	Experienced	RT 0–1 h	32	15.125	<0.001
		RT 2–3 h	30	4.800	0.028
		RT 6–7 h	34	5.452	0.020
		RT 12–13 h	32	0.125	0.724
		CD	35	4.829	0.028
Tr. pretiosum	Naïve	RT 0–1 h	37	4.568	0.033
		RT 2–3 h	30	13.333	<0.001
		RT 6–7 h	34	0.471	0.493
		RT 12–13 h	30	1.200	0.273
		CD	41	0.095	0.758
	Experienced	RT 0–1 h	31	3.903	0.048
		RT 2–3 h	34	11.765	0.001
		RT 6–7 h	33	6.125	0.013
		RT 12–13 h	30	0.533	0.465
		CD	42	0.024	0.876

Parasitoid Response to Synthetic Volatiles Inexperienced *Tr. pretiosum* wasps showed a significant preference to GLV synthetic mixture at all tested concentrations (Fig. 3; 1,000 µl/ml$N$=35, χ2=6.429, P=0.011; 500 µl/ml$N$=35, χ2=4.829, P=0.028; 50 µl/ml$N$=35, χ2=10.314, P=0.001; 5 µl/ml$N$=35, χ2=4.829, P=0.028). In contrast, wasps did not show any attraction to (*E*)-*β*- farnesene at 5 or 50 µl/ml (Fig. 4; 5 µl/ml$N$=35, χ2=0.257, P=0.612; 50 µl/ml$N$=35, χ2=0.029, P=0.866), and preferred control (hexane) over (*E*)-*β*- farnesene at 500 and 1,000 µl/ml (500 µl/ml N=35, χ2=4.829, P=0.028; 1,000 µl/ml$N$=35, χ2=8.257, P=0.004).

Plant Volatile Collection The composition of the volatile mixture released by the different treatments is shown in Table 2. The proportions of compounds in the mixture are depicted in Fig. 5. It should be noted that, as the absolute quantities of the different volatiles cannot be directly compared with the given method, the approach used here is valid for relative differences between compounds at different time

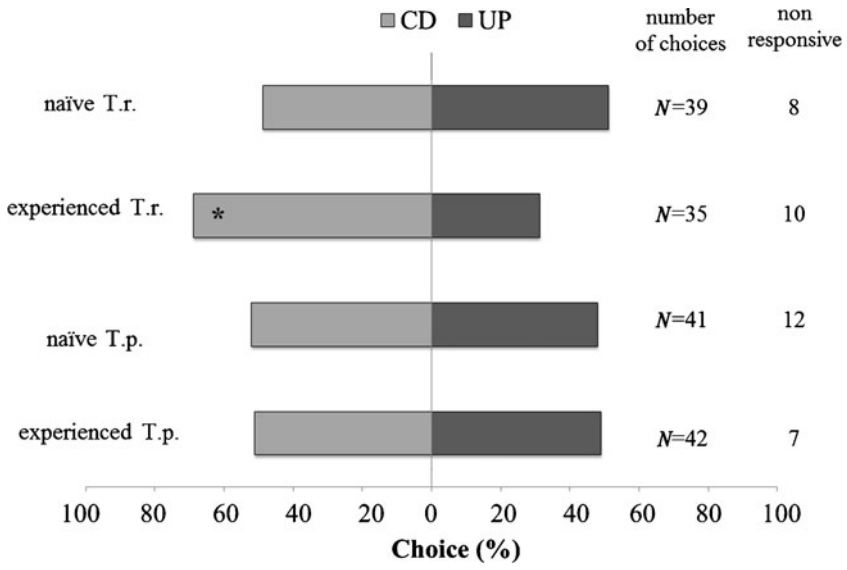


Fig. 2 Response of naïve and experienced *Telenomus remus* and *Trichogramma pretiosum* to volatiles emitted by undamaged plant (UP) and *Spodoptera frugiperda*-caterpillar damaged plant overnight (CD). T.r. = *T. remus*; T.p. = *Tr. pretiosum*. * significant at 5% according to *chi-square*

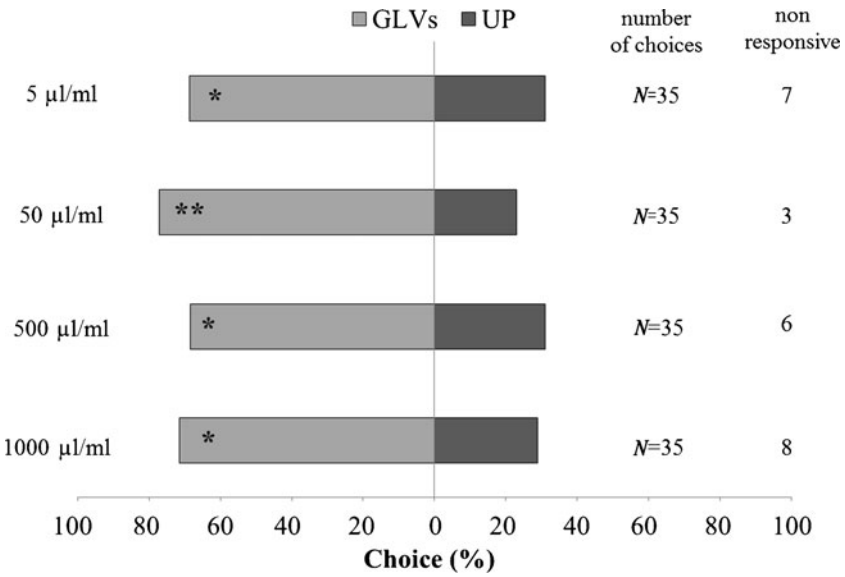


Fig. 3 Response of naïve *Trichogramma pretiosum* to different concentrations of synthetic GLV mixture (GLVs) at 5, 50, 500, and 1,000 µl/ml and hexane as control. * significant at 5% according to *chi-square*; ** significant at 1% according to *chi-square*

points. We observed that GLVs (*Z*)-3-hexenal, (*E*)-2-hexenal, (*E*)-3-hexen-1-ol, and (*Z*)-3 hexenyl acetate are predominantly released by RT 0–1 h, and monoterpenes, such as linalool and β-myrcene are present in small amounts (Fig. 5). On the following time interval, RT 2–3 h, the plants started to emit monoterpenes and aromatic compounds (phenethyl acetate, benzyl acetate, geranyl acetate e indole) in greater amounts, and the homoterpene (3*E*)-4,8-dimetil-1,3,7-nonatriene (DMNT) was abundant in the mixture (Fig. 5). Moreover, at this time interval, sesquiterpene emission commenced. By contrast, emission of GLVs almost ceased, and (*Z*)-3 hexenyl acetate was the only GLV that could still be detected.

At 6–7 h after induction, sesquiterpenes [(*E*)-β-caryophyllene, (*E*)-α-bergamotene, and (*E*)-β-farnesene] were emitted in highest amounts (Fig. 5). All aromatic compounds (except benzyl acetate), DMNT, and monoterpenes also were present in the blend. At this time interval, no GLVs were detected.

The composition of volatiles emitted 12–13 h after induction (induction at night time) was largely similar to the blend at 6–7 h, with exception of geranyl acetate, which was absent. Sesquiterpenes still dominated the mixture, while the proportion of homoterpenes (DMNT) was reduced. Monoterpenes were still present and in higher amounts than at 6–7 h (Fig. 5). In general, overall amounts of volatiles were reduced at 12–13 h compared to emission of RT 6–7 h, with exceptions of (*E*)-β-caryophyllene, which was the major compound among

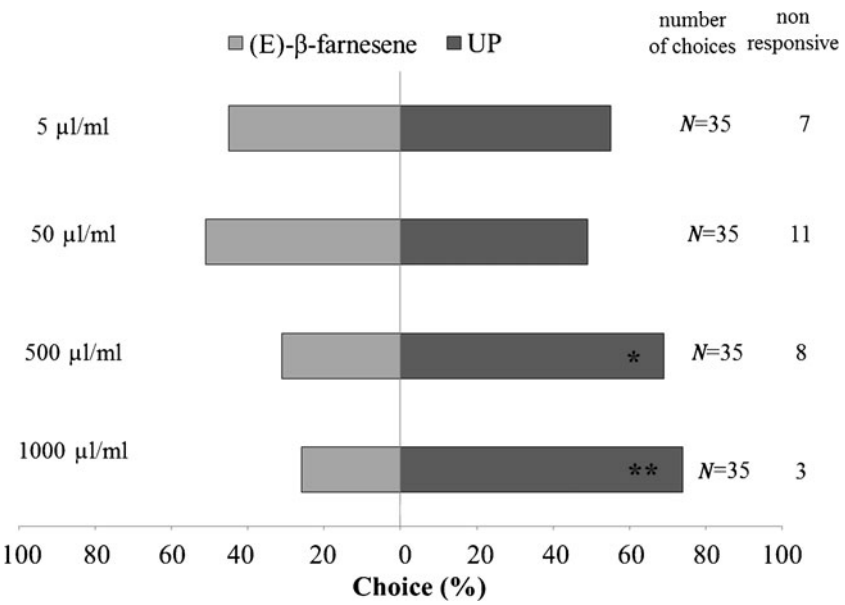


Fig. 4 Response of naïve *Trichogramma pretiosum* to different concentrations of synthetic (E)-β-farnesene at 5, 50, 500, and 1,000 µl/ml and hexane as control. * significant at 5% according to chi-square; ** significant at 1% according to chi-square

Table 2 Amount of volatiles (ng/h) released by *Spodoptera frugiperda* regurgitant- treated (RT) and caterpillar-damaged plant (CD)

Volatile compound	UP	Regurgitant-treated plant				CD
		0–1 h	2–3 h	6–7 h	12–13 h	
Green leaf volatiles (GLVs)						
(*Z*)-3-hexanal		26.3±6.1				122.5±25.1
(*E*)-2-hexanal		103.9±14.9				18.4±2.6
(*E*)-3-hexen-1-ol		10.00±2.3				22.1±2.5
(*Z*)-3-hexen-1-ol acetate		93.9±12.3	5.5±1.5			64.1±10.9
Aromatics						
benzyl acetate			2.00±0.8			5.3±2.0
phenethyl acetate			4.1±1.3	7.0±5.2	2.3±1.1	5.3±2.2
indole			77.7±36.5	69.4±29.0	4.2±2.0	193. 2±78.7
Monoterpenes						
geranyl acetate	6.5±1.2		6.8±2.4	13.9±8.3		8.8±2.5
β-myrcene	8.7±0.3	4.4±0.40	4.0±1.2	2.0±1.3	3.3±0.8	6.7±0.7
linalool	94.0±13.4	18.4±5.0	33.3±9.5	59.2±34.1	41.6±10.8	93.9±25.2
Homoterpenes						
(3*E*)-4,8-dimethyl-1,3,7-nonatriene			180.6±51.1	161.0±78.8	1.3±1.3	30.4±6.0
Sesquiterpenes						
(*E*)-β-caryophyllene			2.6±1.0	13.5±2.8	75.5±5.5	62.3±6.2
(*E*)-α-bergamotene			14.2±3.9	132.0±55.8	38.4±10.7	24.7±5.5
(*E*)-β-farnesene			27.3±9.3	282.2±119.7	53.8±19.5	39.6±12.5
Total	109.2±43.5	261.7±31.2	360.1±99.7	741.2±205.0	224.6±43.4	701.1±146.9

the sesquiterpenes, and β-myrcene. Continuous attack of *S. frugiperda* caterpillars overnight (treatment CD) triggered the emission of a complex volatile blend on the next day, which comprised all chemical groups simultaneously: GLVs, aromatics, homoterpenes, monoterpenes, and sesquiterpenes (Fig. 5). The relative quantities gave rise to a unique pattern of VOCs (Fig. 5).

Discussion

HIPVs can be detected over long distance, making them important signals in the host finding behavior of parasitoids (Vet and Dicke, 1992). While HIPVs can provide specific information about the attacking herbivore, such as species and herbivore stage (Takabayashi et al., 1995; De Moraes et

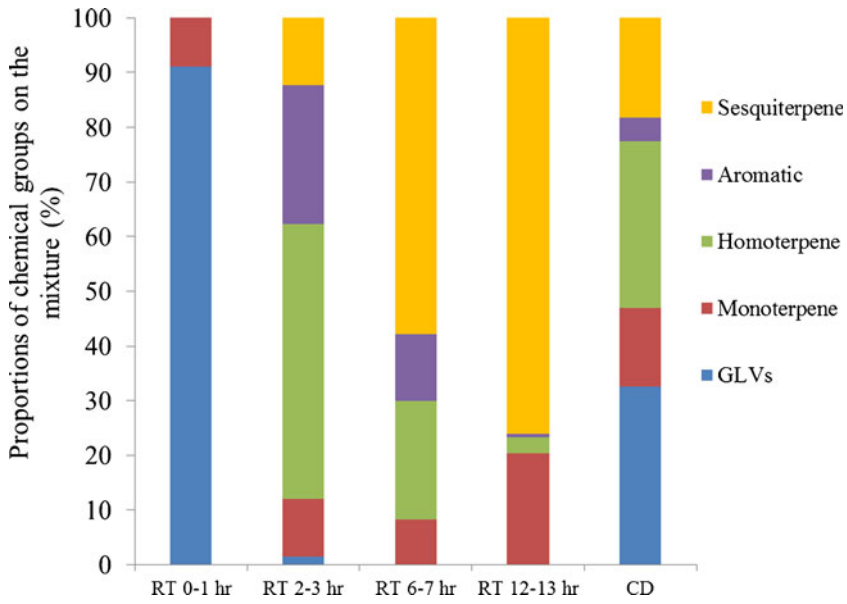


Fig. 5 Proportions of each chemical group present on the mixture released by *Spodoptera frugiperda*-regurgitant induced plant at 0–1 h (RT 0–1 h), 2–3 h (RT 2–3 h), 6–7 h (RT 2–3 h), 12–13 h (RT 12–13 h) and induced by larva feeding overnight (CD)

al., 1998; Mumm et al., 2005), our work shows that they also may serve as indirect cues, possibly giving away the presence of more inconspicuous host stages like eggs. Our results reveal that both generalist and specialist egg parasitoids may use plant volatiles to locate plants with potential hosts. Until now, few other studies have compared the strategies of egg parasitoids with different host ranges, and little has been done regarding their capacity to learn to respond to plant volatiles (Meiners et al., 2003).

The specialist parasitoid, *T. remus*, did not show an innate response towards volatiles emitted by regurgitant-induced maize or by fall armyworm attacked plants (CD). Nevertheless, after experience, *T. remus* was able to learn to associate odors emitted by the artificially induced and attacked plants. This suggests that in a situation where the parasitoid encounters eggs on plants that are attacked by emerging *S. frugiperda* larvae, it can adjust to this situation and use HIPVs as host-finding cues. The plasticity in behavior may be advantageous to the egg parasitoid, as HIPVs may be associated only with eggs under certain environmental situations: In times of high herbivore density, for example, it may be fairly common to find egg masses and larvae occurring simultaneously on the same plants, while in times of low occurrence, this would be less likely. Alternatively, HIPVs might play a role as long-range cues to egg parasitoids. Once wasps find the host community, host odors and subtle oviposition induced cues (Fatouros et al., 2008; Tamiru et al., 2011) from neighboring plants may precisely indicate the presence of the host.

Our results indicate that the multivoltine *S. frugiperda* does not avoid plants infested with conspecifics for oviposition, lending support to the concept that multiple host stages are likely to be present on maize plants under heavy pest pressure.

In contrast to *T. remus*, the generalist *Tr. pretiosum* was attracted innately to volatiles emitted by regurgitant-induced plants at 0–1 and 2–3 h (Fig. 1). These mixtures contained major amounts of GLVs compared to the later time points (Table 2 and Fig. 5). GLVs are thus main candidates for the innate attraction of *Tr. pretiosum*, and our bioassays with synthetic GLVs show that the parasitoid is indeed responding positively to these cues (Fig. 3). Maize larval parasitoids are known to be strongly attracted to fresh damage (Hoballah and Turlings, 2005; Shiojiri et al., 2006; Ngumbi et al., 2009), and other *Trichogramma* species have been shown be reactive to GLVs (Reddy et al., 2002; Romeis et al., 2005; Sen et al., 2005). Interestingly, *Tr. pretiosum* was not attracted to the complete blend elicited by larval feeding, which did contain substantial amounts of GLVs. This suggests that the composition of the HIPVs plays an important role in the attraction of the parasitoid, and some later induced volatiles like aromatic compounds and sesquiterpenes may distort and/or mask the attractiveness of the active volatiles. Additional evidence for the negative impact of these compounds comes from the fact that *Tr. pretiosum* was not attracted to caterpillar induced plants, even after having had a positive oviposition experience (Fig. 2), and from our finding that (*E*)-β-farnesene repelled the parasitoid, at least at high doses. It remains to be determined why *Tr. pretiosum* did not learn the CD blend, although it contained compounds that it was clearly able to perceive and learn. One obvious constraint of the data present here is that volatile collections and olfactometer assays took place in two different locations, and small differences in plant growth conditions may have altered the volatile blends (Gouinguené and Turlings, 2002).

According to the theory proposed by Vet and Dicke (1992), specialist parasitoids should respond innately to volatiles released by host-damaged plants, while generalists should rely more strongly on learning. Our results, however, show the exact opposite: the specialist seemed to rely more strongly on learning, while the generalist showed innate attraction to HIPVs (Fig. 1). This is in accordance with a series of studies showing that innate responses are important in generalists, and specialists retain a high capacity for learning (Dickens, 1999; Shiojiri et al., 2000; Smid et al., 2002; Steidle and Van Loon, 2003; Gouinguené et al., 2005; Hoballah and Turlings, 2005).

Both *T. remus* and *Tr. pretiosum* show innate responses to host odors, such as pheromones and body scales (Beevers et al., 1981; Nordlund et al., 1983; Noldus et al., 1990; Gazit et al., 1996). Similar to HIPVs, these are indirect cues for the egg parasitoids. The innate attraction to such signals may be advantageous because of their relative stability (i.e., pheromones vary very little) and their potential to guide the parasitoids to habitats that are likely to have eggs present within days.

In general, our results are in agreement with findings of Manrique et al. (2005) that studied the egg parasitoid *Anaphes iole* Girault (Hymenoptera: Mymaridae). This parasitoid develops exclusively in hosts of *Lygus* spp. (Hemiptera: Miridae), a group of polyphagous herbivores. In this system, only experienced parasitoids were attracted by volatiles induced by feeding of their hosts. Equally, Moraes et al. (2005, 2009) showed that *Telenomus podisi* (Ashmead) (Hymenoptera: Scelionidae), which is considered a generalist egg parasitoid of pentatomids, is innately attracted by HIPVs released by soybean and chickpea. Taken together, the emerging pattern is that generalist egg parasitoids seem to respond innately to HIPVs, while specialists rely on learning at least when their host is polyphagous. Learning HIPVs could allow specialist parasitoids to exploit odors from different plants and plant genotypes that carry their host (Gouinguené et al., 2001; Degen et al., 2004). This study is, to our best knowledge, the first to compare directly a generalist and specialist egg parasitoid within the same system, and further research comparing several generalist and specialist species is

required to confirm the pattern and explain the observed phenomenon in detail.

Differently from larval parasitoids, egg parasitoids can prevent injuries to crops by killing herbivores before hatching. Our results provide a better understanding on egg parasitoid host searching behavior, and may help to develop techniques that optimize the biological control efficiency of egg parasitoids in the field.

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of α-hydroxynitriles (cyanohydrins), biosynthetically derived from amino acids. They generally co-occur with β-glycosidases that can cleave the glycoside and release cyanide and carbonyl compounds upon damage to the plant. Both cyanide and carbonyl compounds are capable of conferring protection from herbivory and/or pathogens (Harborne, 1982). However, recent evidence has shown that high levels of cyanide (HCN) disrupt the enzymatic activity of polyphenol oxidase (PPO), enhancing the plant susceptibility to fungi (Lieberei, 1988; Ballhorn et al., 2010a; Ballhorn, 2011). Thus, there is a chemical trade-off for plants to resist herbivores or pathogens (Ballhorn et al., 2010a).

There have been several studies that compare the viability and development of the two strains when grown on different hosts (Pashley, 1988a; Quisenberry and Whitford, 1988; Whitford et al., 1988; Pashley et al., 1995; Meagher et al., 2007). In general, the rice strain tended to develop faster than the corn strain when fed on its preferred host, *C. dactylon*, (Whitford et al., 1988; Pashley et al., 1995; Meagher et al., 2007). Comparisons of the survivorship of the two strains on different *Cynodon* hosts were less consistent, with either no significant strain differences observed (Pashley et al., 1995; Meagher et al., 2007) or higher corn strain viability (Whitford et al., 1988). In particular, a past study from this laboratory found that stargrass, a *Cynodon* species frequently found in pastures, was a good host for the development of both strains, with high viability and developmental rates (Meagher et al., 2007).

This paper re-examines the issue of differential host plant usage by the two strains from the perspective of cyanide toxicity. The study was based on unexpected results from feeding studies that appeared to vary from past findings by this laboratory. It was found that a stargrass diet caused a substantial mortality in both strains, with significantly higher levels of larval and pupal mortality observed in the corn strain compared to the rice strain. The possibility that this might be due to high, but transient, levels of cyanogenic compounds produced in cut stargrass was examined. The ramifications of these findings to our understanding of strain-specific host preferences and to the interpretation of past feeding studies are discussed.

Methods and Materials

Plants and Insects Plants were cultivated in glass houses (5×8 m; Lord and Burnham, High Falls, NY, USA) under controlled temperature (22°C), humidity (80%) and photoperiod (14:10-h light/dark). 'Trucker's Favorite' corn and 'NuMex Sahara' bermudagrass plants grew from seeds, while stargrass (*C. nlemfuensis* Vanderyst var. *nlemfuensis* 'Florona') and 'Tifton 85' (a F_1 hybrid pentaploid between the bermudagrass PI 290884 (in the literature as 'Tifton 292') and the stargrass 'Tifton 68') (Burton, 2001) grew vegetatively from stolons in a 4:1 standard substrate (Metro mix 500) that was mixed with leveling sand. Plants were watered once a day and fertilized with 3.1 g/l Miracle Grow water soluble 24:8:16 once a week (The Scotts Company, LLC, Marysville, OH, USA). Two fall armyworm host strains were subjected to feeding experiments with corn and stargrass. The corn strain colony originated from collections of larvae made from corn at the University of Florida Dairy Research Unit, Hague, Alachua Co. FL, USA, while the rice strain colony originated from collections of larvae from pasture grasses at the University of Florida Range Cattle Research and Education Center, Ona, Hardee Co. FL, USA. These two host strains were identified by molecular markers (Levy et al., 2002) as modified by Nagoshi and Meagher (2003), and are currently maintained at the USDA, ARS, CMAVE Insect Behavior and Biocontrol Unit in Gainesville, FL, USA.

Larval Excretion and Adult Body Studies The excreta of 75 4th instars comprising 25 that were fed 'NuMex Sahara', 25 that were fed stargrass, and 25 that were fed 'Tifton 85' were collected after 3–5 h of feeding. Larvae were reared in plastic Sterilite tubs (Sterilite Corporation, Townsend, MA, USA) [26 (d) × 12.1 (h) cm] containing metal screens with a mesh size of 0.7 cm. Plant material was placed on top of the screen, and feces was collected on paper towels that lined the bottom of the tubs. The HCNp of the feces was determined as described below. Larvae were allowed to complete development on their host treatment and the emerged adult bodies were tested for cyanogenesis.

Cyanogenic Potential (HCNp) In order to measure the amount of cyanide released from a given tissue, or cyanogenic potential, the primary blades and the stems from corn, stargrass, 'NuMex Sahara' and 'Tifton 85' were assessed for HCNp during the winter of 2009. Feces from the insect trials, and eight adult moths fed on each diet, also were tested for HCNp.

Cyanogenic potential of plants was quantified by the Lambert procedure (Lambert et al., 1975) as modified by Brinker and Seigler (1989). The plant parts were ground to a fine powder in liquid nitrogen and added to a 10-ml vial containing 0.1 M phosphate buffer (pH 6.8). A 5-ml vial containing fresh 1 M NaOH solution then was placed inside the 10-ml vial, and the 10-ml vial was stoppered tightly and incubated at 37°C overnight. Samples were prepared quickly to avoid loss of HCN. During incubation, HCN released from plant tissue was trapped in the NaOH solution and formed NaCN. After incubation, cyanide was quantified spectrophotometrically (Biotek μQuant microplate, Rockville, MD, USA) using the reagents detailed in Brinker and Seigler (1989). The absorbance was measured at 580 nm. A cyanide standard curve was prepared with

0.02 M solution of NaCN in 0.1 N NaOH (0.980 g NaCN/L). The exact amount of cyanide in that solution was determined by a modified Liebig titration method as described by Brinker and Seigler (1989).

On occasion, plants, animals, or feces that are cyanogenic can yield negative results in tests for cyanogens. The organism may lack cyanogenic glycosides, or may contain cyanogenic glycosides, but not the necessary β-glucosidases needed to release the cyanide. In the latter case, the addition of exogenous β-glucosidase is necessary to quantify the HCNp. Since the cyanogenic glycosides in *Cynodon* spp. are unidentified, a low specificity β-glucuronidase (β-glucuronidase Type H-1 from *Helix pomatia* Müller, Sigma Chemical Co., St. Louis, MO, USA), which releases cyanide from all cyanogenic glycosides, was added to all samples (Brimer et al., 1983; Jaroszewski et al., 2002, Hay-Roe, 2004). For analysis of the HCNp in excreta, we added 500 μl of β-glucuronidase (149 units/ml β-glucuronidase) after thawing the samples in 0.1 M phosphate buffer (pH 6.8). The homogenate then was incubated at 37°C, and HCNp was determined as described above. Eight adult moths fed on each diet were tested for cyanogenesis using samples containing 250 μl of added β-glucuronidase per sample.

Subsamples of ground plant tissue, adult moth bodies, or insect excreta prepared for the cyanogenesis analyses were dried separately at 65°C until they reached a constant dry weight. The dry weight was used to calculate the μg HCNp/g dry weight of plant tissue, adult body tissue, or dry feces. Three replicates each were performed for the ground plant tissue and the insect excreta.

Cyanogenic Capacity (HCNc) Plant tissues were assayed to determine the amount of hydrogen cyanide released per unit of time, a metric known as cyanogenic capacity (HCNc). We were interested only in relative comparisons of HCNc to rank the different plant hosts. Feigl-Anger cyanide test strips were used to quickly and economically provide qualitative information (Feigl and Anger, 1966). The primary blades of 'NuMex Sahara', 'Tifton 85', and stargrass were ground and placed in 5-cm vials with a test strip in each vial. The vial was closed immediately, and patterns of HCN released were tracked every hour for 7 h. Color changes in the test strips were ranked as 0, 1, 2, or 3, according to the intensity of the blue color. Three separate samples were analyzed per plant.

Feeding Experiments A subset of our laboratory colonies was raised on natural host plants for at least three generations before subjecting the neonates to feeding experiments. The corn strain was maintained on greenhouse-grown 'Truckers Favorite' corn, while the rice strain was maintained on greenhouse-grown 'Florona' stargrass. These cohorts were maintained in environmental chambers at 26°C, 75% humidity and a 14:10 h L:D photoperiod.

In total, 120 neonates derived from 30 females of both host strains were raised separately in 1-oz. acrylic SOLO cups with cardboard lids. Fresh food was provided every 8 h during the day. Caterpillars were checked for the presence of head capsule apolysis on a daily basis and the developmental time of each instar was recorded. Head capsule measurements were made with a Wild Heerbrugg M5A microscope and an ocular micrometer. The following formula was used to calculate growth rate:

$$\text{Growth rate} = (\ln(H_f) - \ln(H_i))/t,$$

where H_f is the measurement of the last instar head capsule width; H_i is the measurement of the 1st instar head capsule width; and t is the larval developmental time. The growth rate is approximately the relative size increase on any given day.

The day when pupae formed was recorded as the first day of pupation. Pupal development was complete when the adult moth emerged from the pupal case. Each pupa was weighed 2 d after pupation. Adult sex was recorded and wing length was measured from the base of the wing to the apex using a Vernier caliper (Caliper 2,000 mm) graduated to 0.1 mm. Mass measurements were made with an electronic balance accurate to the nearest 0.1 mg.

Tolerance to Sodium Cyanide In order to determine the toxic effects of cyanide, third, fourth, and fifth instars of the corn and the rice strains were reared on a pinto bean artificial diet (Guy et al., 1985) containing either 0.5% or 0.1% NaCN, which was replaced once a day. Control larvae were fed unamended pinto bean diet. Larvae were raised individually in 1-oz. cups, and body weight, larval developmental time, and mortality rates were recorded.

In order to determine possible habituation to high cyanide concentration, three replicates of 20 newly-molted fourth instars of both strains were fed a 0.5% NaCN-containing pinto bean diet, with replacement of the cyanide-containing diet every 8 hr. When larvae began to molt to the fifth instar, they were transferred to 1% NaCN-containing diet that was replaced every 3 hr during the day (Brattsten et al., 1983).

Statistical Analysis Two-way analysis of variance was used to analyze the feeding experiment, the HCNp in plants, and the HCNp in insect excreta. A Tukey test was used to compare differences between different categories of a discrete variable. Head capsule and HCN measurements were transformed to a natural logarithm to alleviate skewness. A regression analysis was performed to correlate pupal weight and adult size. Data collected in the test of tolerance to NaCN-containing diet were compared to the control pinto bean diet data with the Student paired *t*-test. We used SYSTAT v. 12 (SYSTAT Software, Inc. San Jose, CA, USA) to perform statistical analysis.

Results

Cyanogenic Potential (HCNp) in the Primary Host Plants of Fall Armyworm Each species of plant used in this study had a distinct HCNp. The highest concentration of cyanide was observed in stargrass (1.54 mg HCN/g dry leaf tissue). This level was significantly higher ($F_{3, 40}$=348.2, P<0.001) than the levels found in the bermudagrass line 'NuMex Sahara' (0.16 mg HCN/g dry leaf tissue) or in the hybrid 'Tifton 85' (0.10 mg HCN/g of dry leaf tissue) (Fig. 1). In contrast, corn plants had low HCNp (0.01 mg HCN/g dry leaf tissue) (Fig. 1). Cyanogenic potentials in leaves were higher than in stems for both'NuMex Sahara' and 'Tifton 85' (P<0.05). HCNp in corn leaves and stems and in stargrass leaves and stems were not significantly different (P >0.05) (Fig. 1).

Cyanogenic Capacity (HCNc) in Grasses To estimate the temporal pattern of cyanide release from cut grasses, we determined cyanogenic capacity (HCNc). HCNc is a measure of the release of hydrogen cyanide per unit time (Alonso-Amellot and Oliveros-Bastidas, 2005; Ballhorn et al., 2005, 2010b). Large amounts of cyanide were released from stargrass within seconds after tissue injury, and the cyanide slowly dissipated and became undetectable after 8 hr (Table 1). A similar result was obtained with 'NuMex Sahara', but with lower levels of cyanide released during the first 5 hr. 'Tifton 85' had the lowest HCNc, with detectable cyanide only in the first hour after tissue injury (Table 1). This analysis defined the frequency with which host plant clippings had to be replaced in feeding studies in order to maintain exposure to plant cyanogenic compounds.

Feeding Experiments Laboratory feeding studies were used to compare mortality and developmental growth rates of the two host strains fed either corn or stargrass (Table 2). Both strains had <10% mortality when grown on corn. In comparison, using stargrass as a host resulted in high mortality, with the mortality level for corn strain larvae (77%) significantly higher than that for rice strain larvae (50%) (Z=-2.14, P<0.05). Larvae fed stargrass became dark, lethargic, and flaccid before dying. Pupae developed through the third day of pupation, but then became darker and dried out; some pupae were malformed. The difference in mortality between strains was limited to the larval stage. The few corn strain individuals that emerged to adulthood (N=7) were morphologically normal.

Despite the higher mortality, corn strain larvae had higher growth rates (1.06±0.02 mm/day) (mean ± SE) than rice strain larvae (1.00±0.02 mm/day) ($F_{1,107}$=8.3, P<0.005), with both strains displaying higher growth rates on corn (1.08±0.02 mm/day) than on stargrass (0.98±0.02 mm/day) ($F_{1, 107}$=18.8, P<0.001). Corn strain larvae (20.4±0.5 days) took longer to develop than rice strain larvae (17.6±0.3 days) ($F_{1, 89}$=10.8, P=0.001), no matter which diet was consumed. However, larvae of either strain feeding on corn (20.3± 0.3 days) took 4 days longer to develop than those feeding on stargrass (16.1±0.3 days) ($F_{1, 89}$=59.3, P<0.001). Pupal developmental time in the corn strain (12.0±0.2 days) was 1 day longer compared to the rice strain (11.2±0.2 days) ($F_{1, 74}$=4.7, P=0.03) regardless of diet. When both strains were fed corn they had a longer pupation time than when fed stargrass ($F_{1, 74}$=17.3, P<0.0001). A regression analysis showed that adult size was positively correlated with differences in pupal size in both host strains (P <0.001). The few surviving corn strain adults that had been fed stargrass had significantly longer wings than their rice strain counterparts (P=0.02), while no significant adult size difference was observed when both strains were fed corn (Table 2).

Larval Excretion Studies Fecal matter from larvae feeding on cyanogenic grasses was collected and examined for cyanogenic potential per fecal weight (μg HCNp/g). There was no significant interaction between host strain and plant host (ANOVA, P=0.46), so comparisons are shown between host strains and among grass diets. Corn strain larvae excreted significantly higher levels of cyanogenic compounds than rice strain larvae (Fig. 2a). Roughly equal amounts of cyanogenic compounds were excreted when the corn and rice strains fed on stargrass and 'Tifton 85',

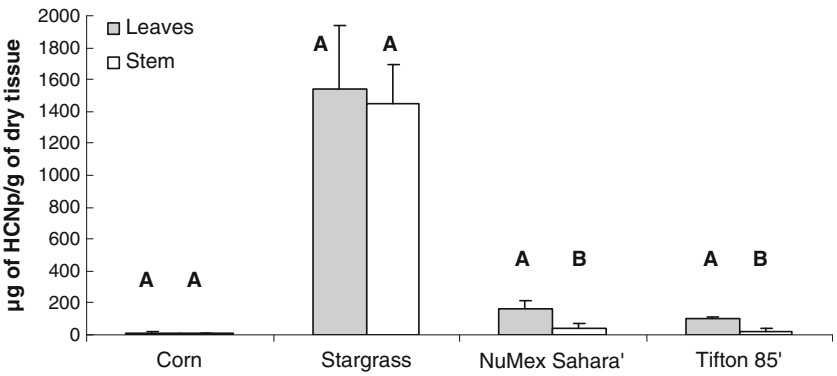


Fig. 1 Spectrophotometric measurement of HCNp from corn, stargrass, 'NuMex Sahara', and 'Tifton 85' leaves and stems. Letters indicate significant differences between plant parts according to *post hoc* analysis (Tukey's HSD P< 0.05). Bars indicate standard error. Mean of three replicates

Table 1 Cyanogenic capacity (HCNc) of injured *Cynodon* spp. plant tissue monitored over an hourly basis with Feigl-Anger strips

Cynodon spp.	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	9 h
Stargrass	3	3	2	2	2	1	1	1	0
'NuMex Sahara'	2	2	1	1	1	0	0	0	0
'Tifton 85'	1	0	0	0	0	0	0	0	0

Progress of hydrolysis: no reaction (0), trace (1), present (2), extreme (3)

and levels for both strains were significantly higher than levels in excretions from larvae consuming the 'NuMex Sahara' diet (Fig. 2b).

Adult moths from both strains were acyanogenic regardless of diet. The addition of β-glucuronidase did not alter the results.

Tolerance to Sodium Cyanide To test more directly whether the two strains differed in their tolerance to cyanide toxicity, feeding studies were performed in which inorganic cyanide (NaCN) was added to an artificial diet. Cyanide concentrations of 0.05% and 0.1% NaCN did not increase larval mortality, but did retard larval development time for both strains. Corn and rice strain 5th instars took a longer time to develop when feeding on either the 0.05% NaCN-diet (corn strain: *t*-test df=25, t=-2.2, P =0.04; rice strain: *t*-test df=28, t=-10.4, P <0.001), or the 0.1% NaCN-diet (corn strain: *t*-test: df=24, t=-3.262, P=0.003; rice strain: *t*-test df=29, t=-8.3, P <0.001) compared to larvae of each strain feeding on the control diet (Table 3). A similar delay in rice strain larval development was reached in the 4th instar for both the 0.05% NaCN-diet (*t*-test df=29, t=-2.8, P=0.009) and 0.1% NaCN-diet (*t*-test df=29, t=-10.0, P<0.001) (Table 3). Neither NaCN treatment produced a significant change in corn strain larval weight. A significant reduction in rice strain larval weight was observed for 3rd instars feeding on 0.1%NaCN (*t*-test df=29, t=2.1, P=0.04) and 5th instars on both NaCN concentrations (0.05%NaCN: *t*-test df=28, t=3.4, P<0.002; 0.1%NaCN: *t*-test df=29, t=4.9, P<0.001) (Table 3).

Substantial mortality was observed at higher concentrations of NaCN. In the habituation study, 58% of corn strain larvae died by the 5th instar on a 0.5% NaCN-diet. The larvae showed evidence of poisoning, such as flaccidness and regurgitation. An additional 19% of the surviving larvae died within 2 days of exposure to the 1% NaCN diet producing a total mortality of 77% by the end of the habituation experiment (Table 4). Only 13 corn strain adults (8 females and 5 males) survived the experiment. Dissection of the dead larvae showed necrosis of the crop and the anterior midgut and a peculiar overgrowth of the salivary glands. In contrast, 80% of the rice strain specimens survived to eclosion. Both strains produced significantly smaller pupae on the high NaCN-diet (Table 4).

Discussion

Corn and stargrass leaves have very different levels of cyanogenic compounds, suggesting that cyanide toxicity may explain elevated fall armyworm mortality when the latter is used as a host plant. Differences in the ability to metabolize or eliminate cyanide may be the physiological basis for the plant host biases exhibited by fall armyworm

Table 2 Percent mortality at different stages of development and adult wing lengths for corn strain (CS) and rice strain (RS) of *Spodoptera frugiperda*. Insects were fed corn or stargrass

Fall armyworm stage	Diet			
	Corn		Stargrass	
	CS($\bar{x} \pm SE$)	RS($\bar{x} \pm SE$)	CS($\bar{x} \pm SE$)	RS($\bar{x} \pm SE$)
Larval mortality (%)	6 [a]	3[a]	47[b]	20[c]
Pupal mortality (%)	0	0	30	30
Total mortality (%)	6[a]	3[a]	77[b]	50[c]
Adult wing length (cm)	1.51±0.01[a]	1.52±0.01[a]	1.50±0.03[a]	1.40±0.02[b]

*Mean comparisons are within strain. Letters indicate significant differences calculated by a *post hoc* (Tukey's HSD; P<0.001) after ANOVA test. Percentage mortality within a column followed by the same letter are not significant different (P>0.05; Z-test)

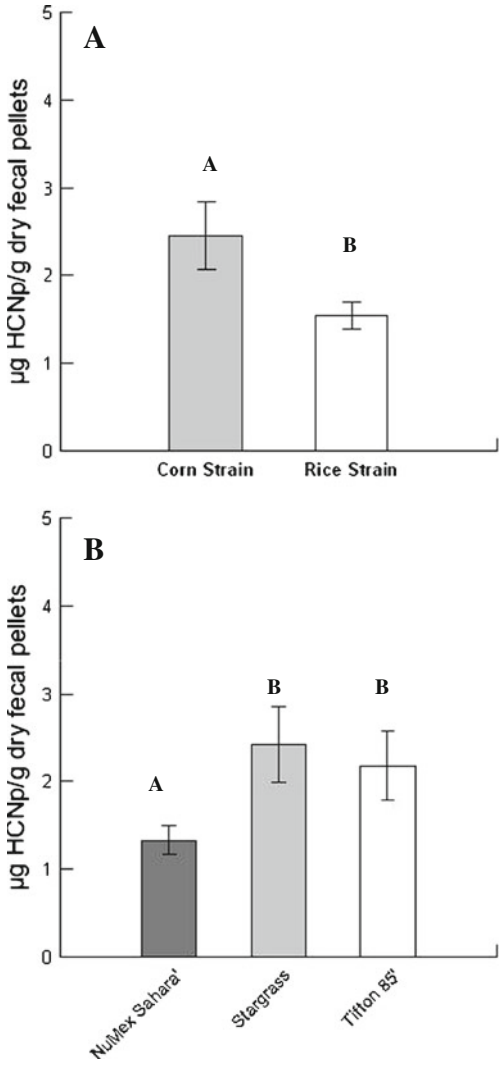


Fig. 2 Spectrophotometric measurement of HCNp from dry fecal pellets of corn and rice strain larvae fed'NuMex Sahara', stargrass, and 'Tifton 85'. **a** shows HCNp for corn strain vs. rice strain ($F_{1,18}$= 6.9, P=0.02). **b** shows HCNp for both strains fed each grass type separately ($F_{2,18}$=3.7, P<0.05). Letters indicate significant differences. Bars indicate standard error. Mean of three replicates

host strains. There are precedents for this mechanism of conferring host plant specificity. Cyanogenesis is a chemical defense mechanism of plants against herbivores, with cyanide inhibiting the electron transport chain in the cell respiratory pathway (Solomonson, 1974). However, some insects and other organisms have adapted to this toxicity and are able to overcome it by sequestration, synthesis, metabolic detoxification, excretion, or regurgitation of the cyanogenic compounds and/or cyanide (Conn, 1981; Nahrstedt, 1988; Jones, 1988; Zagrobelny et al., 2004). Although the function of cyanogenesis in interactions with generalist and specialist herbivores is not fully understood (Gleadow and Woodrow, 2002), there is evidence that plant selection by generalists differs from specialists due to cyanogenic features of the host plant (Engler-Chaouat and Gilbert, 2007; Ballhorn et al., 2010b).

Evidence that the two fall armyworm strains examined here differ in their response to cyanide exposure is based on two main results. The corn strain has significantly higher mortality levels when fed inorganic cyanide than the rice strain. The relative tolerance of the rice strain to cyanide toxicity is consistent with its preference for *Cynodon* species (Pashley, 1988a; Meagher et al., 2007) that produce substantial concentrations of cyanogenic compounds. The second observation is the statistically significant difference in cyanide levels in the fecal excretions of corn strain larvae when grown on cyanogenic grasses (Fig. 2). This indicates that there are physiological differences between strains for the processing, elimination, or tolerance of ingested cyanogenic compounds.

These results appear to contradict an earlier study from our laboratory that did not demonstrate increased mortality, or any aberrations in the development of fall armyworm, when grown on a stargrass diet (Meagher et al., 2007). We believe this apparent conflict is due to methodological differences in the two studies. In the previous study, plant cuttings used for diet, including stargrass, were replaced every 1–2 days. Given the lability of the cyanogenic compounds in cut plant tissue (Table 1), the availability of cyanide in the diets in our earlier study was probably intermittent at best. In the current study, the stargrass was replaced with fresh cuttings every 8 hr, thereby providing a more consistent exposure to cyanide.

In addition, Meagher et al. (2007) reared larvae in a chamber where the plant cuttings were placed on a water-saturated filter paper (to prolong the quality of the plant material). Because cyanide is water-soluble, this procedure had the potential to leach cyanide from the diets, thereby reducing the levels ingested. To avoid such leaching in this study, rearing containers were kept dry, and fresh plant cuttings were added more frequently. Cyanogenesis in stargrass may also vary substantially, depending on a number of environmental factors (Aguilera et al., 1982a,b, 1984). In particular, seasonal variation in cyanide concentration is a common trait of cyanogenic plants (Cooper-Driver et al., 1977; Kaplan et al., 1983; Jones and Rammani, 1985; Gebrehiwot and Beuselinck, 2001; Hay-Roe and Nation, 2007).

Finally, the young leaves of cyanogenic plants generally contain larger quantities of cyanide than mature leaves and stems (Conn, 1981; Goodger et al., 2007; Hay-Roe and Nation, 2007; Ballhorn et al., 2008). This phenomenon has been associated with higher concentrations of soluble protein in young leaves, compared to mature leaves (Ballhorn et al., 2008). In our present study, we found that leaves and stems of stargrass did not differ in their HCNp. Aguilera et al. (1985) reported that under certain conditions, related to

Table 3 Life history parameters at different stage of development of corn strain (CS) and rice strain (RS) of *Spodoptera frugiperda.* Insects were fed a control pinto bean diet, or the same diet amended with 0.05% or 0.1% NaCN

Fall armyworm stage	Control diet		0.05% NaCN		0.1% NaCN	
	CS($\bar{x} \pm SE$)	RS($\bar{x} \pm SE$)	CS($\bar{x} \pm SE$)	RS($\bar{x} \pm SE$)	CS($\bar{x} \pm SE$)	RS($\bar{x} \pm SE$)
3rd instar						
LDT (days)	3.0±0.0	3.0±0.0	3.0±0.0	3.0±0.0	3.0±0.0	3.0±0.0
Larval weight (mg)	54.0±1.9	50.8±2.4	47.3±2.6	51.2±2.0	53.2±2.4	43.6±2.4*
4th instar						
LDT (days)	3.0±0.2	2.3±0.1	2.9±0.2	2.5±0.1*	2.8±0.1	3.3±0.1**
Larval weight (mg)	142.5±3.7	128.5±3.1	133.8±4.4	132.9±3.4	134.3±4.3	134.2±2.7
5th instar						
LDT (days)	6.0±0.3	5.3±0.1	6.4±0.2*	6.2±0.1**	6.6±0.2*	6.0±0.03**
Larval weight (mg)	239.2±5.0	225.2±3.0	240.1±5.0	209.3±3.6*	230.9±3.7	203.3±3.7**

Asterisks indicate significant differences from the control calculated by *t*-test * $P<0.05$, ** $P<0.001$. (LDT larval developmental time)

the amount of fertilizer and the testing period after plant fertilization, cyanide concentration in leaves and stems of *C. nlemfuensis* does not vary. Therefore, comparisons of feeding studies, even from the same laboratory, could be problematic if the potential variability in cyanide levels is not considered.

Cyanide poisoning in larvae generally was characterized by flaccidness in the body and regurgitation behavior, with dead larvae displaying crop necrosis and overgrowth of the salivary glands. Similar phenotypes including flaccid bodies and dried, dark and lacerate pupae, also were observed in the dead larvae and pupae that had been fed the stargrass diet. These observations suggest that cyanide poisoning may account in part for the reduced viability observed on stargrass. We also note that sublethal levels of NaCN in the diet produce significant delays in developmental time and larval weight (Table 3). Previous research showed that pinto bean artificial diet slows larval and pupal developmental time and that rice strain fed on this diet experienced lower growth rates than the corn strain (Quisenberry and Whitford, 1988; Hay-Roe, personal observations), suggesting that this variability is related to the pinto bean diet.

Table 4 Pupal weight and percent mortality after the habituation test of corn (CS) and rice strain (RS) of *Spodoptera frugiperda.* Insects were fed a control pinto bean diet, or the same diet amended from 0.5% to 1% NaCN

	Habituation			
	Control		1%	
	CS($\bar{x} \pm SE$)	RS($\bar{x} \pm SE$)	CS($\bar{x} \pm SE$)	RS($\bar{x} \pm SE$)
Pupal weight (mg)	214.2±4.1	201.4±2.5	168.2±7.2**	153.1±3.0**
Mortality (%)	0	2	77 **	20 **

Asterisks indicate significant differences from the control calculated by *t*-test ** $P<0.001$. Mortality rates differences were calculated by *Z*-test, $P<0.001$

The observation that the two strains differ in the amount of cyanide excreted while feeding on stargrass is intriguing but difficult to interpret (Fig. 2). The data indicate that the corn-strain is more efficient at eliminating cyanide, yet it is also the most susceptible to its toxic effects. These results suggest that excretion is not the primary mechanism used by either strain to detoxify cyanogenic compounds and that the strain difference observed may simply reflect the higher sensitivity of the corn strain to the toxic effects. In other words, the higher HCN levels in the corn strain feces may be an incidental consequence of physiological abnormalities caused by cyanide toxicity. The possibility that the metabolism of the toxic cyanogenic compounds involves insect digestive enzymes or formamide hydrolyase produced by pathogens of cyanogenic plants (Fry and Munch, 1975) cannot be discarded.

Adult moths were acyanogenic in both host strains, and this suggests that cyanogenic glycosides are not sequestered by the larvae and are not used as a protective mechanism by fall armyworm adults, as occurs in some other Lepidoptera (Franzl and Naumann, 1985; Zagrobelny et al., 2004). The larvae do regurgitate gut contents as a defensive mechanism against predators, but regurgitates from fall armyworm fed cyanogenic compounds were not analyzed for cyanogenesis.

In summary, evidence is presented that plant cyanogenic glycosides might play a significant role in defining the host range of the two fall armyworm strains. Differences in cyanide levels were observed in the host plants, and the two strains differed in their sensitivity to cyanogenic compounds and in their capacity to metabolize and/or eliminate these compounds. Host plant specificity could be the force driving the divergence and incipient speciation of the two host strains.

Our analysis of cyanogenic capacity has clarified factors in the variable and often contradictory results reported in previous feeding studies. The way that plant material is

prepared and the frequency with which new material is added to the diet can significantly affect the levels of cyanide exposure in feeding studies, with potential to confound experimental observations of developmental rate, mortality, and larval behavior.

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on flowers during nectar consumption may help to protect plants against herbivores and flower-parasites (Yano, 1994; Bleil et al., 2011). However, they are usually floral antagonists that consume nectar without pollination and/or aggressively defend flowers against true pollinators (Galen, 1983; Lach, 2005; Junker et al., 2010a). It has been suggested that the exposure to nectar-thieving ants selects for the emission of ant-repellent floral scents as well as other types of defenses (Junker et al., 2011a). Therefore, floral scents may have both attractive and defensive properties serving as potent filters that promote floral visitation by mutualists but restrict other visitors including antagonists (Kessler et al., 2008b; Raguso, 2008a; Junker et al., 2010b).

Floral scents comprise complex mixtures of chemical compounds that belong to different chemical classes. Terpenoids and benzenoids are two prominent classes of compounds emitted from flowers of most plant species (Knudsen et al., 2006). Recently, two studies proposed a functional dichotomy of these classes: terpenoids, especially monoterpenoids, have been suggested to function as defense compounds, and benzenoids have been supposed to be attractants (Junker and Blüthgen, 2010b; Schiestl, 2010). Schiestl (2010) investigated the correlation between the evolution of floral scents and the chemical communication of insects by comparing volatile organic compounds that are emitted by flowers with those used by insects for inter- and intra-specific communication. He found a strong overlap between individual monoterpenoids that occur in floral scents and those that function in the communication systems of herbivorous insects. On the other hand, benzenoids have been found both in floral scents and as communication substances in taxa of pollinating insects. Meanwhile, Junker and Blüthgen (2010b) conducted a meta-analysis and compared the responses of obligate (usually mutualistic) and facultative (usually antagonistic) flower visitors to a variety of floral scent compounds documented in the literature; they showed that monoterpenoids have strong repellent properties against facultative flower visitors, such as ants and herbivorous animals, while benzenoids are attractants for obligate flower visitors, such as members of many pollinator taxa. The results of these studies indicate that the dual role of floral scent bouquets arises from the complex blend of attractive and defensive components (compare to Kessler et al., 2008a) that may derive from different biosynthetic pathways.

In this study, we focused on the defensive properties of floral scents, testing the hypothesis that floral terpenoids mediate repellent effects against ants. We inhibited terpene biosynthesis in flowers of *Phlox paniculata* (Polemoniaceae) and tested the effect of the inhibited scent bouquet vs. the uninhibited control on the behavior of *Lasius niger* ants. For inhibition, we used fosmidomycin and mevinolin, substances that inhibit the pathways of monoterpene and sesquiterpene biosynthesis, respectively (Dudareva et al., 2005; Schie et al., 2006). The flower odor of *P. paniculata* is composed mainly of monoterpenoids and benzenoids, and to a lesser extent of sesquiterpenoids and fatty acid derivatives (Andersson et al., 2002). Thus, this species is well suited to test our hypothesis about the defensive roles of floral terpenoids. An advantage of this approach is that inhibitor treatment of flowers should reduce the emission of terpenoids without influencing the composition of the benzenoids and fatty acid derivatives. In addition to the experiments with ants, we also examined the attractiveness of treated and untreated flowers to typical pollinator taxa.

Methods and Materials

Study Organisms *Phlox paniculata* plants (Polemoniaceae, variety Peppermint Twist) were obtained from a plant nursery (Gärtnerei Boock, Jena, Germany). The plants were reared without applying insecticides or other plant protection products, neither in the plant nursery nor after purchasing. Plants were kept outdoors and watered daily prior to the start of the experiment in July and August 2009. When we performed the experiments, all plants were flowering. *Lasius niger* (Hymenoptera, Formicinae) ants from three different colonies were collected in the field (Würzburg, Germany) in July 2009. Workers and brood were kept in plastic containers (25×16×12 cm) filled with garden soil as a substrate in a climate chamber (14:10 h. L:D, 26°C/19°C, with a constant relative humidity of 50%.). The walls were coated with a thin layer of Fluon (Whitford, Diez, Germany) in order to prevent ants from escaping. Ants were fed with a sugar solution (30%) offered on filter paper and tuna meat; Additionally, water was provided in tubes sealed with cotton wool. The substrate in the containers was watered regularly, to stay moist.

Inhibition of Floral Terpene Biosynthesis We inhibited terpene biosynthesis with the pathway-specific inhibitors fosmidomycin (Invitrogen, Carlsbad, CA, USA) and mevinolin (Sigma-Aldrich, Taufkirchen, Germany) (Dudareva et al., 2005). Fosmidomycin inhibits 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), an enzyme in the plastidic methylerythritol phosphate pathway of terpene biosynthesis, which usually provides the substrate for monoterpenoid formation (Schie et al., 2006). Mevinolin inhibits 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), an enzyme of the cytosolic mevalonate pathway of terpene biosynthesis, which generates the precursors for sesquiterpene synthesis (Schie et al., 2006). Fosmidomycin was dissolved in distilled water to a final concentration of 10 mM. Prior to application, the mevinolin lactone was converted into the open acid form (sodium salt) as described

in Kita et al. (1980) in order to increase water solubility. The mevinolin sodium salt was dissolved in distilled water to a final concentration of 1 mM.

Individual flowers were picked in the evening (around 8 pm) and immediately placed in vials filled with aqueous sucrose solution (5%) and subject to one of the following treatments: (1) control *C*: no addition; (2) addition of the inhibitors fosmidomycin and mevinolin *FM*: fosmidomycin and mevinolin were added to the sucrose solution to a final concentration of 75 μM each. We used both inhibitors simultaneously because, even if the plastidic or cytosolic pathway is blocked, terpene precursors may be transported between these compartments (Dudareva et al., 2005; Schie et al., 2006). Scent sampling and olfactometer trials with ants were performed the next morning between 12 and 18 h after treatment. Flowers were used only if they were in good condition and of normal appearance.

Floral Volatile Collection and Analysis Volatile organic compounds emitted by *Phlox paniculata* flowers treated with fosmidomycin and mevinolin (*N*=4) or untreated controls (*N*=4) were collected in a dynamic headspace collection system located in a growth chamber set at 20°C, 55% relative humidity and 85±5 μmol m^{-2} sec^{-1} photosynthetically-active radiation. At 16 h after treatment application, the flowers were placed individually in 1 l glass desiccators (Schott, Mainz, Germany) tightly closed with a glass lid. Charcoal-filtered air entered the desiccators via a valve at 0.7 $lmin^{-1}$. After being in contact with the plant, the air exited the glass cylinder through a Super Q trap (filled with 20 mg Super Q, ARS, Gainesville, FL, USA), positioned in an opening in the lid to absorb flower volatiles. Volatiles were collected for 2 h starting at noon. Afterwards, the volatile compounds trapped were desorbed by eluting the filters with 150 μl dichloromethane containing nonyl acetate as an internal standard (10 ng μl^{-1}). Samples were stored at -20°C until analysis with GC-MS and GC-FID.

Qualitative and quantitative determination of *P. paniculata* volatiles was conducted using an Agilent 6890 Series gas chromatograph coupled to an Agilent 5973 quadrupole mass selective detector (interface temp.: 270°C; quadrupole temp.: 150°C, source temp.: 230°C, ionization potential: 70 eV) or to a flame ionization detector operated at 300°C. The constituents of the volatile bouquet were separated with a DB-5MS column (Agilent, Santa Clara, CA, USA, 30 m×0.25 mm× 0.25 μm) and He (MS) or H_2 (FID) as carrier gas at 1 ml min^{-1}. A 1 μl sample was injected splitless at an initial oven temperature of 40°C that was kept constant for 2 min and then increased to 155°C with a gradient of 7°C min^{-1}. Thereafter, temperature was increased by 60°C min^{-1} to reach a maximum of 300°C that was kept constant for 3 min.

Most compounds were identified by comparison of their retention times and mass spectra to those of authentic standards. However, four compounds [(*E*)-β-ocimene, lilac aldehydes A and B, and lilac alcohol D] were identified solely by comparison of mass spectra to those of reference spectra in Adams (Adams, 2007) and the Wiley and National Institute of Standards and Technology (NIST) libraries. The absolute amount of all compounds was determined based on their FID peak area in relation to the area of the internal standard using the effective carbon number (ECN) concept as described by Scanion and Willis (1985).

In addition to the scent sampling as described above, we sampled the flowers that were used as scent source for olfactometer trials immediately prior the tests by using SPME (solid phase microextraction) fibers consisting of 100 μm polydimethylsiloxane (Supelco, Bellefonte, PA, USA). These scent samples were used to check for variation in the effect of inhibitors and the natural variation in scent production in *P. paniculata*. For logistical reasons, we used two SPME fibers, but each fiber was used for both control and treated flowers on different days in order to compare the proportional emission of individual volatiles between samples. Statistical comparisons (described below) showed that the individual fiber had no influence on the volatile analysis. Flowers were placed in 1 l glass desiccators covered tightly with glass lids that contained a Teflon plug with a small hole into which the SPME fiber for volatile collection was inserted. Floral headspace volatiles collected in the glass vial were trapped for 20 min. The adsorbed volatiles were analyzed afterwards by directly inserting the SPME fiber into the injector of the gas chromatograph. GC/MS analysis and product identification was performed as described above.

Olfactometer Trials For olfactometer trials, we used the same setup as described in detail in Junker and Blüthgen (2008). In the four-pointed star shaped arena of a Petterson olfactomter (Pettersson, 1970) (length from tip to tip: 330 mm, depth: 15 mm), four distinct odor fields were created in which ants can move and respond to different odors. The arena was covered with a glass plate. Each arm of the arena was connected to an odor supply. Openings for air inlet and outlet were obstructed with aluminum mesh to prevent ants from escaping. A constant airflow of 200 ml min^{-1} was pumped into each of the four arms of the olfactometer, maintained with separate flow meters (Analyt, Müllheim, Germany). Two flowers in a glass vial were placed into a desiccator. Charcoal purified air that previously passed through a bottle filled with distilled water entered the desiccator containing the flowers, and was then pumped into the two opposite arms of the arena. Charcoal filtered moistened air containing no odor was pushed into the two other arms of the arena as a control. A neon lamp (220 V, 11 W) illuminated the arena equally. A central hole (10 mm diam) in the arena allowed the air to flow out of the arena.

Treatments *C* and *FM* were repeated six times for olfactometer trials. In each replication, 8 - 10 trials with 10 ants

each were performed, resulting in a total of 136 olfactometer trials (*C*: *N*=58, *FM*: *N*=78) with 1360 ants. In each trial, ten *L. niger* workers were simultaneously released in the arena, and the number of ants in odor and control fields was counted after 60, 90, 120, and 150 s. The mean number of ants in the odor and control fields at the four time points was calculated, and for each trial the mean difference of number of ants *D* in odor and control fields per 150 s period was calculated (*D*=mean number in odor fields−mean number of ants in control fields) as the basis for the statistical analyses. The positions of odor and control fields within the arena were altered after each 150 s period (odor fields were transformed to control fields and *vice versa*), and ants were replaced by a new group of ten ants. Repellence of floral scents was indicated by a significantly lower mean number of ants in odor fields than in control fields (i.e., $D<0$).

Olfactometer Trials with Individual Substances The results of the previous experiments suggested that linalool—one of five monoterpenoids that are emitted by flowers of *P. paniculata*—primarily triggered the ants' aversion towards these scent bouquets (see below). To verify this result, we performed olfactometer trials with synthetic linalool (Merck, Darmstadt, Germany, ≥ 97% linalool) in six different concentrations. Linalool was dissolved in light paraffin oil (AppliChem, Darmstadt, Germany), and 25 µl of the paraffin-scent solution were applied on a filter paper. Concentrations were chosen in a way that the mass of linalool applied on the filter paper corresponded to the mean hourly linalool emission [ng h^{-1}] of two control flowers and to 1/10x, 10x, 100x, 1000x, and 10,000x the hourly emission. Filter papers were placed in an upright position in a desiccator. A filter paper treated with light paraffin oil only served as the control in another desiccator. Desiccators were connected to the olfactometer setup, and experiments with *L. niger* workers were performed as described above. Per concentration, we performed 24 trials as described above with 10 ants each. Additionally, four further substances [benzaldehyde (Fluka, Munich, Germany, ≥ 99%), eugenol (Merck, Darmstadt, Germany, ≥ 99%), (*Z*)-β-ocimene (mixture of isomers SAFC, Hamburg, Germany, GC-MS analysis revealed that only (*Z*)-β-ocimene is included plus limonene), 2-phenyl acetaldehyde (Sigma-Aldrich, Munich, Germany, ≥ 90%)], which were also detected in the scent bouquet of *P. paniculata*, were used for olfactometer trials in the same dilution series. Note that the flowers emitted (*E*)-β-ocimene, but not (*Z*)-β-ocimene. Per substance and concentration, twelve trials with 10 ants each were conducted.

Visitation by Flying Flower Visitors In order to check the attractiveness of *P. paniculata* flowers to flying flower visitors, 3 flowers of treatments *C* and *FM*, each, were placed in glass vials filled with water next to a blooming *Buddleja davidii* (Scrophulariaceae) shrub. These trials were repeated eight times with a set of 3 control- and 3 treated flowers 18 h after the flowers had been picked and treated. Flower visiting frequency and duration as well as the identity of flower visitors were noted.

Statistical Analysis Scent composition (emission per substance [ng h^{-1} $flower^{-1}$]) of control flowers (*C*) and flowers treated with inhibitors (*FM*)—as revealed after volatile collection using Super Q traps—were compared using "*random forest*", a machine learning algorithm that assigns samples to predefined groups (*C* and *FM*) in multiple iterations and estimates the importance of each compound for a correct classification (Breiman, 2001). This bagging method has been applied successfully in other ecological studies to classify samples based on volatile- and/or community-composition, and its advantages and applicability (i.e., it calculates the importance of each variable for a correct classification independently of all others but also considers multivariate interactions with the others while not over-fitting the data) have been demonstrated (Prasad et al., 2006; Ranganathan and Borges, 2010; Junker et al., 2011b). For our statistical analysis, we drew n_{tree}=100,000 bootstrap samples with m_{try}=4 variables randomly selected at each node. To receive optimal results, it is suggested to set m_{try} equal or close to the square root of number of variables (i.e., in this case number of volatiles=16) used for the classification (Breiman, 2003). The implementation of *random forest* in the statistical software R (R Development Core Team, 2011) returns a confusion matrix that summarizes the results of the classification as well as the variable importance *E* for a correct classification. Substances that are differently emitted by the groups (*C* and *FM*), and thus contribute heavily to the correct classification, receive high positive *E*-values, whereas substances that are uninformative for the classification or work against it (i.e., the substances are emitted equally by the groups) receive negative *E*-values. The results from the random forest analysis were confirmed by Mann–Whitney *U*-tests comparing the emission [ng h^{-1} $flower^{-1}$] of individual substances as well as the total emission of control (*C*) and treated flowers (*FM*).

Ant responses *D* towards control bouquets (*C*) in the olfactometer-arena were compared to responses *D* to treated bouquets (*FM*) with a *t*-test to evaluate whether the changes in the floral scent bouquet resulted in changes in ant behavior. Furthermore, responses *D* to scents of control (*C*) and treated flowers (*FM*) were tested individually against the null hypothesis with a paired *t*-test, i.e., against $D=0$ assuming an equal number of ants in odor and control fields of the arena.

In order to assess how individual floral scent compounds influenced the ants' choices in the olfactometer arena, we pooled the *D* values obtained from individual sets of control (*C*, *N*=6) and treated flowers (*FM*, *N*=6) to a mean $\overline{D}$ (each $\overline{D}$ value included 8-10 150 s periods), and fitted linear

models with the proportion of individual floral scent compounds within the bouquet—as revealed after volatile collection using SPME fibers—as the explanatory variable and $\overline{D}$ as the response variable. Since individual SPME fibers may have different adsorption properties to different volatiles, we used the SPME fiber as an additional explanatory variable and tested for both the interaction between SPME fiber and proportional emission of substances and the influence of the SPME fiber on the responses itself.

All statistical analyses were performed with the statistical software R (R Development Core Team, 2011).

Results

Floral Scent Composition of Control and Treated Flowers Dynamic headspace collection with Super Q filters and subsequent GC-MS analysis revealed that flowers of *Phlox paniculata* emitted 16 substances from four classes (Table 1). Qualitatively, the scent bouquets of control (*C*, $N=4$) and treated (*FM*, $N=4$) plants were the same, i.e., the same substances were found in control and treated flowers. However, we found strong differences in the quantitative composition of the samples, mainly caused by a strong reduction in terpenoid emission (Table 1). Accordingly, *random forest* analysis correctly assigned all of the bouquets of inhibitor-treated flowers to the treated group (*FM*, class error=0%) and three out of four control bouquets to the control group (*C*, class error=25%) indicating that one control sample also contained fewer terpenoids than the others (Table 1). Four out of six terpenoid substances (including monoterpenoids and the sesquiterpene germacrene D) received positive *E*-values, indicating that the bouquets of control and treated flowers can be best discriminated by these volatiles, which was partly supported by the Mann–Whitney *U*-tests (Table 1). Non-terpenoid substances received negative *E*-values (except for methyl eugenol) indicating that they were not differently emitted by control and treated flowers; accordingly no differences were detected by Mann–Whitney *U*-tests (Table 1). Total scent emission [ng h^{-1} $flower^{-1}$] was not significantly different in control and treated flowers (Table 1).

Olfactometer Trials In total, we performed 136 olfactometer trials (*C*: $N=58$, *FM*: $N=78$) with a total of 1360 ants. Ant responses to scents of untreated (*C*) and treated (*FM*) flowers were different (Welch *t*-test: $t_{128.1}=2.8$, $P<0.01$, Fig. 1): the natural floral scent bouquet repelled ants (*t*-test: $t_{57}=3.62$, $P<0.001$, Fig. 1), while the ants responded neutrally towards treated flowers (*FM*) with only low amounts of terpenoids emitted (*t*-test: $t_{77}=0.26$, $P=0.80$, Fig. 1).

For each floral bouquet tested, the mean proportion of ants in odor fields of the olfactometer in relation to control fields $\overline{D}$ correlated negatively with the proportion of linalool in the odor, determined from volatile collection with SPME fibers (Linear model: $F_{11}=5.42$, $P=0.042$). Neither the interaction term proportion of linalool * fiber ($F_9=1.60$, $P=0.24$) nor fiber as an explanatory variable influenced this result ($F_{10}=1.01$, $P=0.34$). We did not find similar relationships between the proportion of other substances emitted by *P. paniculata* and the ants' responses in the olfactometer arena ($F_{11}\leq4.26$, $P\geq0.066$).

Olfactometer Trials with Individual Substances As suggested by the negative correlation between the ants' responses and the proportion of linalool within the scent bouquets, ants were repelled by linalool when individually applied in the olfactometer trials (*t*-test: $t_{139}=2.61$, $P=0.01$), and the ants' responses in the olfactometer arena were negatively correlated to the mass of linalool applied on filter papers (Pearson's product–moment correlation: $t_{138}=3.25$, $P<0.01$, $r^2=0.07$). However, benzaldehyde and 2-phenyl acetaldehyde likewise repelled ants in the olfactometer trials (*t*-test: $t_{71}\geq2.11$, $P\leq0.038$), and their masses applied to the filter papers also influenced the ants' behavior in the olfactometer arena (Pearson's product–moment correlation: $t_{70}\geq2.29$, $P\leq0.025$, $r^2\geq0.07$). None of the three substances was repellent to ants in trials where the mass of substance applied to the filter paper matched the hourly emission of these substances (*t*-test: $t_{11/23}\leq1.62$, $P\geq0.13$). Ants' responses were not different towards these repellent substances at either concentration (ANOVA: $F_{2,33}<1.71$, $P>0.2$). Eugenol and (*Z*)-β-ocimene did not repel ants (*t*-test: $t_{71}\leq0.67$, $P\geq0.51$).

Visitation by Flying Flower Visitors Irrespective of the treatment, flowers were highly frequented by hoverflies (*Episyrphus balteatus*, $N=29$) and occasionally by other dipterans ($N=9$), honeybees (*Apis mellifera*, $N=2$) and one butterfly species (*Aglais urticae*, $N=1$). Control flowers received slightly more visits (mean±*SE*: 7.96±1.65 visits/h) than treated flowers (5.71±1.36 visits/h), but this trend was not significant ($t_7=1.65$, $P=0.14$). The duration of individual visits was slightly, but not significantly shorter on control flowers (mean±*SE*: 13.56±5.51 s) than on treated flowers (17.88±6.34 s) ($t_{34}=0.51$, $P=0.61$). Note that *E. balteatus* accounted for 70.7% of all visits, but as this species is not able to collect nectar that is located in deep nectar tubes in *P. paniculata*, only pollen was collected.

Discussion

Our results partly support the hypothesis formulated by Schiestl (2010) and Junker and Blüthgen (2010b) that the monoterpenoids of floral scent mixtures have defensive roles. The flowers of *Phlox paniculata* emit various volatiles

Table 1 Scent emission of *Phlox paniculata* flowers and results of *random forest* analysis. For each compound, values give median and range of the emission [ng h^{-1} $flower^{-1}$] of substances from control (C, *N*=4) and treated (*FM*, *N*=4) flowers. Substances are listed by chemical class and then by retention time. Substances marked by an asterisk were identified using commercially available standard compounds; others by comparison of mass spectra with those in a reference library (see Methods for details). Classification of the floral scent bouquets emitted by control flowers (C) and flowers that were treated with fosmidomycin and mevinolin (*FM*) based on quantitative emission rates of individual substances is given in the confusion matrix resulting from *random forest* analysis. This matrix shows the number of correctly assigned scent bouquets to either control or treated flowers and class errors. Also given is the variable importance *E* of each floral scent compound, positive values indicate compounds that strongly contributed to a correct assignment to the groups. *P*-values for comparison of the total emission and the emission of individual substances in control and treated flowers using Mann–Whitney *U*-test are also given

Compound	Control **C**		Treatment **FM**		E	p
[ng hr^{-1} $flower^{-1}$]	Median	Range	Median	Range		
Fatty acid derivatives						
Nonanal*	1.61	0.95–2.15	2.55	0.75–3.28	-2.79	0.31
Decanal*	0.61	0.00–2.22	1.21	0.00–1.53	-11.65	0.77
sum	2.04	1.32–4.36	3.88	0.75–4.59		0.49
Benezoid compounds						
Benzaldehyde*	37.48	14.93–91.61	23.99	3.39–38.22	-2.24	0.31
2-Phenyl acetaldehyde*	27.47	14.13–62.88	30.60	2.11–49.74	-7.98	0.66
2-Phenyl acetonitrile*	2.20	1.16–4.89	2.34	1.29–3.50	-3.91	0.69
Eugenol*	1.78	0.18–4.20	1.23	0.44–1.48	-6.72	0.89
Methyl eugenol*	28.65	11.18–47.50	19.67	11.57–19.77	17.59	0.31
Methyl isoeugenol*	2.28	0.00–4.63	1.13	0.68–2.26	-3.26	0.89
sum	102.13	52.81–199.92	78.67	21.05–113.96		0.34
Monoterpenoids						
(E)-β-Ocimene	22.37	13.13–28.49	10.09	4.43–14.59	22.08	0.11
Linalool*	7.41	2.92–12.69	0.98	0.00–1.47	50.51	0.03
Lilac aldehyde A	1.54	1.29–3.70	1.24	0.00–1.64	-8.87	0.34
Lilac aldehyde B	1.74	1.26–4.51	0.30	0.00–0.63	56.57	0.03
Lilac alcohol D	0.00	0.00–0.41	0.00	0.00–0.83	-3.05	1.00
sum	33.27	18.60–49.39	12.95	4.94–17.96		0.03
Sesquiterpene						
Germacrene D*	3.04	0.67–4.99	0.43	0.23–0.67	28.32	0.06
Unknown compounds						
Unknown 1	0.00	0.00–0.70	0.00	0.00–0.00	0.00	0.45
Unknown 2	0.66	0.00–0.74	2.96	0.00–7.04	-4.97	0.56
Total hourly emission [ng hr^{-1} $flower^{-1}$]	140.56	86.49–259.08	93.87	32.51–137.82		0.34

Confusion matrix	**C**	**FM**	class error
C	3	1	0.25
FM	0	4	0.00

of different chemical classes. Nearly a quarter of the natural blend consists of monoterpenoids, and these had a strong repellent effect on *Lasius niger* workers. However, in flowers treated with fosmidomycin and mevinolin, the emission of monoterpenoids was strongly reduced. The scents of the treated flowers were no longer avoided by ants in the olfactometer trials, which indicates that the flower odor bouquet had lost its ant repellent function. The emission of linalool, one of the five detected monoterpenoids, was most strongly affected by the treatment with the pathway specific inhibitors: the treated flowers emitted 7.5 times less linalool than the control flowers on average. The negative correlation between the proportion of linalool within the bouquet and ant responses suggests that this substance may be especially responsible for the aversion of ants to the flower odors. This result was supported by olfactometer trials with pure linalool, which alone had a repellent effect on *L. niger* ants. Linalool was shown earlier in olfactometer trials and other experimental setups to be repellent against various ant species including *L. niger* (Cane, 1986; Junker and Blüthgen, 2008, 2010a).

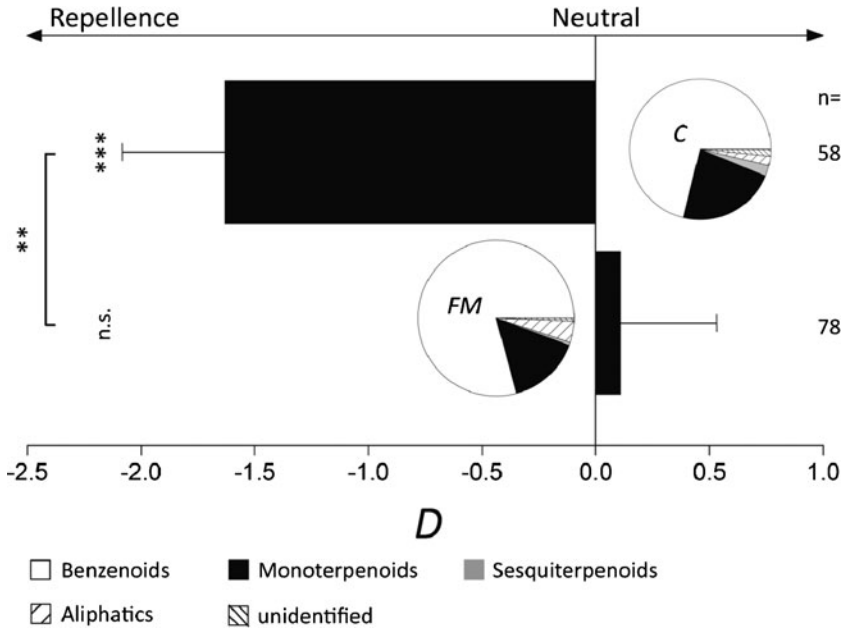


Fig. 1 Results of olfactometer trials. Responses of *Lasius niger* workers (*D*, mean±*SE*) to natural scents of *Phlox paniculata* flowers (control *C*, upper bar) or to scents of flowers that were treated with a mix of fosmidomycin and mevinolin (*FM*, lower bar). *D* is defined as the mean number of ants in odor fields of the olfactometer minus the mean number in control fields. Responses to *C* and *FM* flowers were compared to each other by a *t*-test and each response to *C* or *FM* flowers was tested against the null hypothesis, i.e., equal numbers of ants in scented and neutral fields of the oflactometer-arena ($D=0$). Significance levels of *t*-tests are indicated as n.s. = not significant, ** $P<0.01$, *** $P<0.001$. Pie charts illustrate the mean proportional composition of the scent bouquets emitted by control flowers (*C*) and flowers treated with fosmidomycin and mevinolin (*FM*) as evaluated after dynamic headspace collection

However, two benzenoids, benzaldehyde and 2-phenyl acetaldehyde, also strongly repelled ants when individually applied in olfactometer trials. These and the results from other studies that showed ant repellence due to benzenoids (Junker and Blüthgen, 2010a) challenge the functional dichotomy of benzenoids and monoterpenoids suggested by Schiestl (2010) and Junker and Blüthgen (2010b). While the olfactometer trials with flowers as scent sources suggest that linalool alone elicited the ants' aversion, the results using synthetic scents suggest that in addition to linalool, the benzenoids benzaldehyde and 2-phenyl acetaldehyde also have the potential to repel ants.

The question arises why the modified scent bouquets with a reduced proportion of monoterpenoids including linalool but a similar total emission did not repel ants, although benzaldehyde and 2-phenyl acetaldehyde were still present in comparable amounts. A possible explanation may be that none of the scents was repellent to ants at concentration that matched the natural emission when tested in isolation. Nonetheless, the negative correlation between the proportion of linalool within the samples and the ants' responses still suggests importance of this substance in ant repellence.

The present study demonstrates that the specific terpene pathway inhibitors, fosmidomycin and mevinolin, are effective tools for modifying scent compositions for experiments on the behavior of flower visitors. To inhibit the biosynthesis of terpenoids, the application of both fosmidomycin and mevinolin is recommended, as the transport of isopentenyl pyrophosphate (IPP) from the cytosolic (MVA) pathway into the plastids may compensate for the lack of plastidic pathway product. The transport of IPP the other way around, i.e., from the plastid into the cytosol, is suggested as well (Dudareva et al., 2005; Schie et al., 2006). In a previous study where only fosmidomycin was used to alter herbivore-induced volatile emission from leaves of lima bean and brussels sprouts, significant monoterpenoid inhibition could be achieved only in lima bean, whereas no inhibition of sesquiterpenoids could be attained in either species (Mumm et al., 2008). Consequently, the responses of the predators that are usually attracted to herbivore-induced volatiles of the tested plant species were shown to be weaker in lima bean plants that emitted less monoterpenoids, but no information was available about responses to monoterpenoids in brussels sprouts or about sesquiterpenoids in either species (Mumm et al., 2008).

Despite the enormous complexity of floral scent bouquets, their ecological function has been studied mostly in pollinator attraction. Recently, several alternative functions of floral volatiles have been proposed and investigated (reviewed by Raguso, 2008b). Among these, one important function is the ability to act as floral filters that serve simultaneously as attractive and repellent signals, and thus are able to shape the spectrum of flower visitors, both

mutualists and antagonists (Kessler and Baldwin, 2007; Kessler et al., 2008b; Junker and Blüthgen, 2010b; Junker et al., 2010b; Schiestl et al., 2011). The attractive function of flower odors often has been attributed to whole bouquets, but also to individual components.

Although naïve responses to full odor blends often are stronger than responses to individual compounds within these blends (Stringer et al., 2008), learned responses often are based on "key odorants" within bouquets that serve as recognition cues for a multi-component blend (Laloi et al., 2000; Dötterl et al., 2006; Riffell et al., 2009; Reinhard et al., 2010). The recognition and learning of individual scent compounds associated with rewards is a key process in non-specialized flower-insect interactions (Smith et al., 2006), and the scent compounds involved are widely distributed in flowers of different plant species (Wright and Schiestl, 2009). For example, *Helicoverpa armigera* moths have the ability to use various widespread substances as learned cues to locate nectar bearing flowers (Cunningham et al., 2004). Given this behavior, the scents that insects use to find and consume floral resources of plants need not have any co-evolved links to them, which may explain the commonness of certain floral scent compounds across many plant families (Knudsen et al., 2006). The taxa studied here, *L. niger* and *P. paniculata*, occur sympatrically in North America, but it is unlikely that the repellent effect of the *P. paniculata* flower scent on *L. niger* ants is a result of a species-specific adaptation. Linalool is among the most commonly occurring floral volatiles (Knudsen et al., 2006), and its role in pollinator attraction (Dobson, 2006) but also in defense against antagonists (Junker and Blüthgen, 2010b) has been demonstrated. Thus, in the same way that various scents can be used for associative learning by non-specialized pollinators, floral scent compounds may be universally defensive against facultative and often antagonistic flower visitors (Junker and Blüthgen, 2010b). This notion is supported by the fact that many terpenoids are toxic to arthropods (Gershenzon and Dudareva, 2007).

Our results demonstrate that the presence of terpenoids—especially the monoterpene alcohol linalool—in sufficient amounts triggers the negative responses by ants towards the floral scent of *P. paniculata*. However, other compounds may also be responsible for this defensive function and need to be evaluated in further studies. This result, reinforces those of several recent studies (Kessler and Baldwin, 2007; Kessler et al., 2008b; Junker et al., 2010b; Schiestl et al., 2011) that suggest that the chemical complexity of floral scent bouquets evolved as a response to both mutualists and antagonists.

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and existing studies suggest that the response is less predictable than for trees. For instance, phenolics did not change under elevated CO_2 in hairy bittercress, common groundsel, or corn spurrey (Hartley et al., 2000), increased in alfalfa (Agrell et al., 2004) and ginger (Ghasemzadeh et al., 2010), increased or did not change in soybean (O'Neill et al., 2010) and increased or decreased in birdsfoot trefoil (Bazin et al., 2002; Goverde et al., 2004).

When damaged, many plant species increase levels of defensive compounds. Such chemical induction is thought to be an integral component of adaptive anti-herbivore chemistry (Karban and Baldwin, 1997). Phenolics are among the most inducible compounds in response to damage (Kaplan et al., 2008). Unfortunately, little is known about the effect of elevated CO_2 on the inducibility of plant defenses. Two opposing hypotheses seem reasonable to us: 1) that increased carbon availability will result in increased inducibility, due to increased substrate availability, or 2) that decreased nitrogen content will result in decreased inducibility, due to decreased availability of nitrogen-rich RNA and enzymes required for rapid induction.

While scores of studies have addressed the effect of elevated CO_2 on constitutive chemical defenses, to our knowledge only seven studies have addressed the effect of elevated CO_2 on the inducibility of plant defenses. Induction of phenolics was not affected by elevated CO_2 in aspen (Lindroth and Kinney, 1998) or oak (Rossi et al., 2004), nor was induction of flavonoids in soybean (O'Neill et al., 2010), phenolics and cyanide glycosides in birdsfoot trefoil (Bazin et al., 2002), or saponins and flavonoids in alfalfa, and terpenoid aldehydes in cotton (Agrell et al., 2004). To our knowledge, only two studies have detected an effect of elevated CO_2 on defense induction in response to damage. Bidart-Bouzat et al. (2005) observed an increase in induction of glucosinolates in *Arabidopsis thaliana* under elevated CO_2, and Zavala et al. (2008) observed a decrease in inducibility of soybean protease inhibitors under elevated CO_2.

In addition to chemical defenses, most plants possess mechanical defenses. Trichomes ("leaf hairs"), which are present in virtually every plant family (Marks, 1997), are the most common mechanical defense. Like phenolics, trichomes can reduce oviposition, consumption, growth, and survivorship of insect herbivores (Simmons and Gurr, 2005; Plett et al., 2010). Despite being identified by Pritchard et al. (1999) over a decade ago as a high priority subject, we are aware of only a handful of studies that address the effect of elevated CO_2 on trichomes. Existing studies report either a decrease (Bidart-Bouzat et al., 2005; Lake and Wade, 2009) or no change (Lau et al., 2008; Riikonen et al., 2010; Vannette and Hunter, 2011) in trichome density under elevated CO_2.

Brassica rapa (oilseed rape or canola) possesses both phenolic and trichome defenses and belongs to a genus that has been the subject of much research on plant-herbivore interactions. In addition, *B. rapa* is ranked behind only soybean as the most important source of vegetable oil in the world (Raymer, 2002). In this study, we asked whether growth under elevated CO_2 affects constitutive levels and inducibility of phenolics, and density of trichomes in leaves of *B. rapa*.

Methods and Materials

Plant Growth *Brassica rapa* seeds were germinated in mid-June in the greenhouse at the University of Michigan Biological Station (UMBS) and transplanted 2 day later to 6-in pots containing topsoil. Plants were watered daily or as necessary, and were inspected periodically for insect herbivory; only undamaged plants were used. Plants were fertilized every 2 week with 100 ml full strength Peter's 20-20-20 to avoid nutrient stress. Three days after transplanting, 3 plants were randomly placed into each of 12 0.5-m^3 open-topped chambers maintained at ambient CO_2 (379±10 ppm) and each of 12 chambers maintained at elevated CO_2 (744± 12 ppm). Chambers were arranged into blocks, with each block containing one ambient and one adjacent elevated chamber. Within blocks, CO_2 treatment was randomly assigned to chambers. The chambers were located in a field near the UMBS greenhouse and were designed to maintain natural temperature and light conditions as much as possible (Karowe, 2007). CO_2 content was elevated by dispensing 100% CO_2 into the inlet port of an outlet blower connected by a dryer hose to the base of each elevated chamber. CO_2 levels were monitored by an infrared gas analyzer approximately every 25 min and CO_2 flow was manually adjusted by a flowmeter when needed.

All surviving plants (N=27 at each CO_2 level, including at least 2 from each chamber) were harvested after 40–44 day in the chambers. Constitutive and induced phenolic levels were quantified from opposite halves of the second fully expanded leaf. A razor blade was used to remove one randomly chosen half of the leaf, leaving the midrib intact. At the same time, a garlic press was used to damage 9 cm^2 in the middle of the 1st, 3rd, and 4th fully expanded leaves. This method allowed us to standardize the amount of damage across plants. Twenty-four hours later, the remaining half of the 2nd leaf was harvested. Immediately after removal, each leaf half was flash frozen in liquid nitrogen, stored at −80°C for 1 week, and freeze-dried. We believe that, relative to the usual approach of using different plants for constitutive and induced levels, this paired design is a more powerful method for detecting induction.

Total Phenolics After each leaf half was ground to a fine powder, 20 mg of tissue were washed x 3 with 1 ml diethyl

ether containing 1% (v/v) acetic acid to remove chlorophyll. After each wash, samples were centrifuged at 4,600*g* for 10 min and the ether was removed. The resulting decolorized pellet was resuspended in 0.5 ml of 70% acetone to extract total phenolics, sonicated on ice for 30 min, and centrifuged at 4,600*g* for 10 min. This was repeated x 3, and the 3 supernatants were combined and brought to a total volume of 1.5 ml using 70% acetone. Total phenolics were quantified using the Folin-Denis method. To each reaction tube, 0.5 ml of supernatant, 1 ml Folin-Denis reagent (75 mM sodium tungstate, 1.3 mM phosphomolybdic acid, 2.8% phosphoric acid), and 1 ml 2 N sodium carbonate were added. Samples were incubated for 1 hr at room temperature for color development. Sample absorbency was determined at 725 nm against a blank of deionized water (FAO/IAEA 2000). All samples were run in duplicate. Total phenolic levels were quantified by comparison to a standard curve prepared from tannic acid.

Simple Phenolics To determine levels of simple phenolics, tannins were removed by adding 100 mg of polyvinyl polypyrrolidone, which binds complex phenolics such as condensed dimers and tannins, to a 100×12 mm test tube containing 1.0 ml deionized water and 0.5 ml of the total phenolic extract (FAO/IAEA 2000, modified by M. Grant). After 15 min at 4°C, the extract was centrifuged at 4,600*g* for 10 min, and the supernatant, presumably containing only simple phenols (phenolic acids, alcohols and amines, flavonoids, anthocyanogens, catechins, and flavanols), was collected. The Folin-Denis method was used to determine relative concentration of simple phenolics. Complex phenolics were calculated as the difference between total and simple phenolics measured from the same extract.

Carbon and nitrogen contents were determined for each ground leaf sample using a Perkin Elmer CHN Elemental Analyzer (Waltham, MA, USA).

Trichomes From a different set of plants than those used for C, N, and phenolic analyses (N=115 plants at ambient CO_2 and 119 plants at elevated CO_2), trichomes on the lower leaf surface from two 0.9 cm^2 disks removed from the distal tip of the second fully expanded leaf were counted under a dissecting microscope. Trichome counts from the two disks per leaf were averaged.

Statistical Analysis The experimental design was a split-plot with repeated measures, with CO_2 as whole plots, and damage as the repeated measure, since carbon, nitrogen, C:N ratio, and phenolics were measured on opposite (pre- and post-damage) halves of the same leaf (Littell et al., 1998). Variables were analyzed with a *Type III ANOVA* (PROC MIXED) with CO_2 level and damage as fixed effects, and block and the CO_2*block interaction as random effects (SAS Institute, 2000). To further determine whether phenolics were inducible at each CO_2 level, paired *t*-tests were used to compare phenolic levels in the half of a leaf sampled immediately before damage and the opposite half of the same leaf sampled 24 hr after damage. To determine whether CO_2 affected inducibility, the magnitude of change in phenolic levels was compared between CO_2 levels by independent samples *t*-tests. Normality of inducibility values was verified by *Kolmogorov-Smirnov* tests and equality of variances was verified by F_{max} tests.

To determine whether leaf percent N influenced constitutive phenolic levels and/or magnitude of phenolic induction, these two variables were regressed against leaf N and C:N ratio.

Trichome density was measured only on undamaged plants. Since variances were unequal and data were not normal, mean trichome density was compared between CO_2 levels by a *Mann–Whitney U* test. The proportion of leaves with at least one trichome was compared between CO_2 levels by a *chi-square* test.

Results

Nutritional Quality Under elevated CO_2, nitrogen content of *B. rapa* leaves decreased significantly (by 16%, from 2.70 to 2.29% of dry weight, means for constitutive and induced combined) and C:N ratio increased significantly (by 20%, from 15.47 to 18.11) (Table 1). Carbon content averaged 40.17% and was not affected by CO_2 level. No measured component of leaf nutritional quality was affected by damage, or showed a significant CO_2*Damage interaction.

Constitutive Phenolics When both constitutive and induced levels were considered together, growth of *B. rapa* under elevated CO_2 did not result in a significant change in levels of simple, complex, or total phenolics (in each case, P>0.2 for the overall CO_2 effect; Table 1). However, when only constitutive levels were considered, elevated CO_2 resulted in a 17% increase in complex phenolics (t=2.83, P=0.007) and a 14% increase in total phenolics (t=2.76, P=0.008). In contrast, constitutive levels of simple phenolics were not affected by growth of *B. rapa* under elevated CO_2 (t=1.65, P=0.10).

Inducibility of Phenolics When all plants were considered together, damage resulted in significant increases in complex (by 20%) and total phenolics (by 14%), but did not affect levels of simple phenolics (see the Damage effect in Table 1). ANOVA analysis detected an effect of CO_2 on inducibility, indicated by the significant CO_2*Damage interaction for each phenolic variable in Table 1. Paired *t*-tests at each CO_2 level revealed that simple, complex, and total

Table 1 Constitutive and induced levels (as a percent of leaf dry weight; mean ± SD) of nitrogen, carbon, C:N ratio, and simple, complex, and total phenolics in *Brassica rapa* leaves grown under ambient and elevated CO_2

Variable	CO_2 level	Damage	Mean ± SD [a]	Effect	Significance[b]
Nitrogen	Ambient	Constitutive	2.67±0.47	CO_2	**<0.001**
		Induced	2.73±0.58	Damage	0.42
	Elevated	Constitutive	2.25±0.38	CO_2*Damage	0.90
		Induced	2.33±0.43		
Carbon	Ambient	Constitutive	39.99±1.42	CO_2	0.33
		Induced	40.04±1.40	Damage	0.74
	Elevated	Constitutive	40.29±1.03	CO_2*Damage	0.95
		Induced	40.37±1.04		
C:N ratio[c]	Ambient	Constitutive	15.50±3.20	CO_2	**<0.001**
		Induced	15.44±3.91	Damage	0.55
	Elevated	Constitutive	18.35±3.06	CO_2*Damage	0.81
		Induced	17.87±3.27		
Simple phenolics	Ambient**[d]	Constitutive	0.35±0.04	CO_2	0.83
		Induced	0.39±0.05	Damage	0.40
	Elevated	Constitutive	0.37±0.06	CO_2*Damage	**0.028**
		Induced	0.34±0.10		
Complex phenolics	Ambient***	Constitutive	0.58±0.10	CO_2	0.26
		Induced	0.77±0.14	Damage	**<0.001**
	Elevated**	Constitutive	0.68±0.15	CO_2*Damage	**0.015**
		Induced	0.75±0.14		
Total phenolics	Ambient***	Constitutive	0.94±0.12	CO_2	0.36
		Induced	1.15±0.17	Damage	**<0.001**
	Elevated*	Constitutive	1.05±0.16	CO_2*Damage	**0.008**
		Induced	1.11±0.19		

[a]Sample size is 27 for each CO_2 × Damage combination

[b]*P*-values from *ANOVA* analyses; significant effects are in bold

[c]Data were normalized by log transformation prior to analysis

[d]Induction was significant at this CO_2 level by a paired *t*-test; $P<0.05$ (*), $P<0.01$ (**), or $P<0.001$ (***); see Fig. 1

phenolics were all more inducible under ambient CO_2 than under elevated CO_2. In response to damage, simple phenolics increased by 11% under ambient CO_2 (*paired* $t=3.03$, $P=0.006$), but did not change under elevated CO_2 (*paired* $t=1.31$, $P>0.2$; Fig. 1); this difference in inducibility was highly significant ($t=5.51$, $P<0.001$). Induction of complex phenolics occurred at both CO_2 levels, but was stronger for plants grown under ambient CO_2 than for plants grown under elevated CO_2. Complex phenolics increased by 33% under ambient CO_2 (*paired* $t=9.39$, $P<0.001$) but by only 12% under elevated CO_2 (*paired* $t=3.67$, $P=0.002$); this difference in inducibility was also highly significant ($t=4.10$, $P<0.001$). Similarly, total phenolics increased by 24% under ambient CO_2 (*paired* $t=9.90$, $P<0.001$) but by only 6% under elevated CO_2 (*paired* $t=2.11$, $P=0.045$). Like that of simple and complex phenolics, inducibility of total phenolics was greater at ambient CO_2 ($t=4.47$, $P<0.001$; Fig. 1).

Correlations between Nitrogen, C:N, and Phenolics CO_2 level affected the strength of correlation between measures of leaf nutritional status (N and C:N) and phenolics. For plants grown under ambient CO_2, neither N nor C:N was significantly correlated with constitutive level or inducibility of simple, complex, or total phenolics. However, for plants grown under elevated CO_2, constitutive levels of simple phenolics were positively correlated with leaf C:N ratio ($R=0.55$,

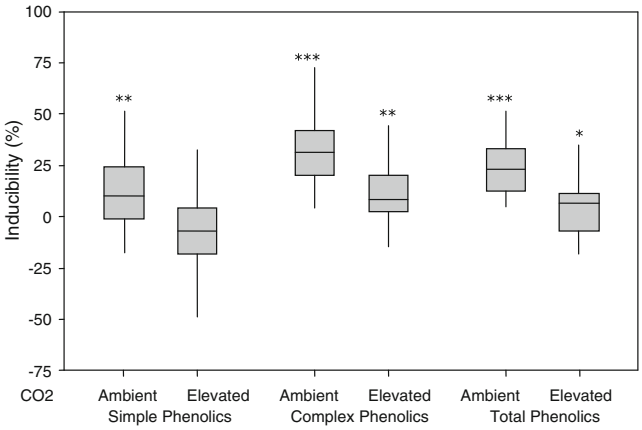


Fig. 1 Inducibility (% change during 24 hr after damage) of simple, complex, and total phenolics in *Brassica rapa* leaves[a]. [a]*Shaded* areas include the first through third quartiles, *horizontal lines* indicate medians, and *vertical lines* indicate the range. *Asterisks* denote significant induction at a given CO_2 level (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). In all cases, inducibility was significantly greater at ambient CO_2 than at elevated CO_2

d.f. = 26, *P*=0.003; Fig. 2) and negatively correlated with leaf nitrogen (*R*=–0.53, *d.f.* = 26, *P*=0.005). In contrast, the inducibility of simple phenolics was positively correlated with leaf nitrogen (*R*=0.54, *d.f.* = 26, *P*=0.004; Fig. 3) and negatively correlated with leaf C:N ratio (*R*=–0.46, *d.f.* = 26, *P*= 0.015). When all plants were considered together (but not at either CO_2 level alone), the inducibility of complex phenolics was not significantly positively correlated with leaf nitrogen (*R*=0.24, *d.f.* = 53, *P*=0.084; Fig. 4) nor significantly negatively correlated with leaf C:N ratio (*R*=–0.23, *d.f.* = 53, *P*= 0.095). When inducibility was expressed as an absolute rather than percent change, the inducibility of complex phenolics was positively correlated with leaf nitrogen (*R*=0.33, *d.f.* = 53, *P*=0.014) and negatively correlated with leaf C:N ratio (*R*=–0.34, *d.f.* = 53, *P*=0.013). In no case was leaf carbon content significantly correlated with any phenolic measure.

Trichomes Growth of *B. rapa* under elevated CO_2 resulted in a significant increase in constitutive trichome density (*Mann–Whitney U*=12,362, *d.f.* = 1, *P*=0.015). Under ambient CO_2, there were 28.2±5.6 trichomes/cm^2 of leaf area, and this value increased by 57% to 44.3±6.4 trichomes/cm^2

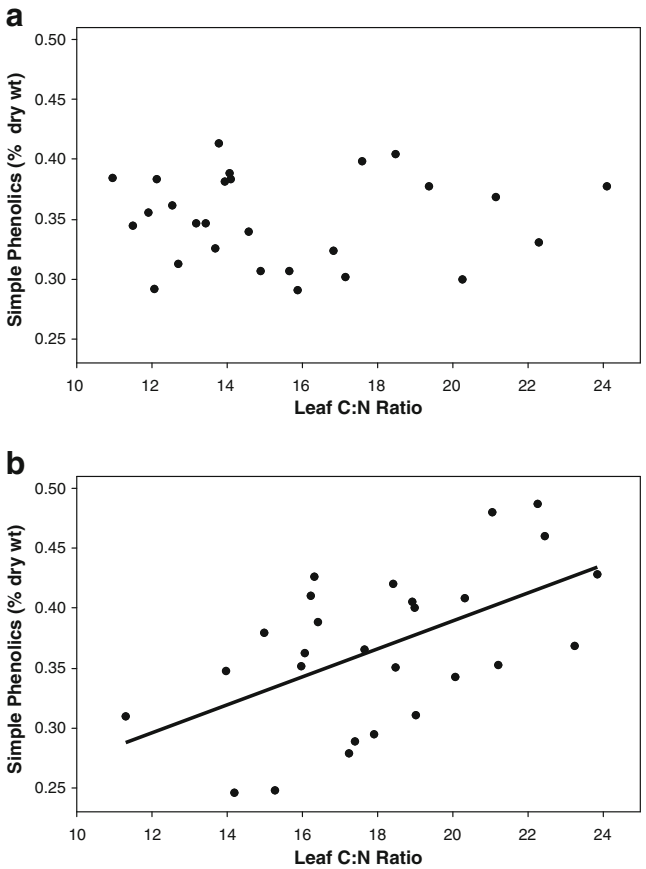


Fig. 2 Relationship between C:N ratio of *Brassica rapa* leaves and constitutive levels of simple phenolics (as a% of leaf dry weight) for plants grown at (**a**) ambient CO_2 (*R*=0.01, *P*>0.9) and (**b**) elevated CO_2 (*R*=0.68, *P*=< 0.001)

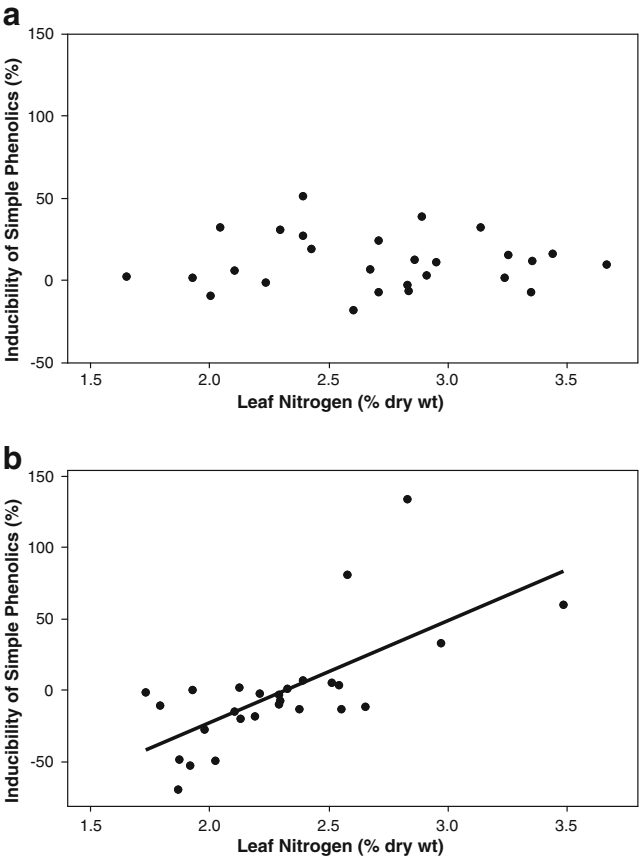


Fig. 3 Relationship between nitrogen content of *Brassica rapa* leaves and inducibility (% change during 24 hr after damage) of simple phenolics for plants grown at (**a**) ambient CO_2 (*R*=0.01, *P*>0.9) and (**b**) elevated CO_2 (*R*=0.54, *P*=0.004)

under elevated CO_2 (Fig. 5). In addition, a significantly lower proportion of leaf discs contained zero trichomes under elevated CO_2 (46.2%) than under ambient CO_2 (61.7%) (χ^2= 5.67, *d.f.* = 1, *P*=0.017). Trichome density was also nearly significantly more variable at ambient CO_2 than at elevated CO_2 (*Levene's statistic* = 3.66, *P*=0.057; Fig. 5)

Discussion

The results of this study indicate that rising atmospheric CO_2 can affect both chemical and mechanical defenses of *Brassica rapa*. To our knowledge, this is only the second study to demonstrate that inducibility of a chemical defense is diminished under elevated CO_2.

The observed 16% decrease in nitrogen and 20% increase in C:N ratio of *B. rapa* leaves under elevated CO_2 are similar to the mean changes reported for non-legume C_3 plants (Cotrufo et al., 1998), herbaceous plants (Zvereva and Kozlov, 2006), and other *Brassica* species (Karowe et al., 1997). Given the observed increase in *B. rapa* C:N ratio under elevated CO_2, it is reasonable to expect that constitutive levels

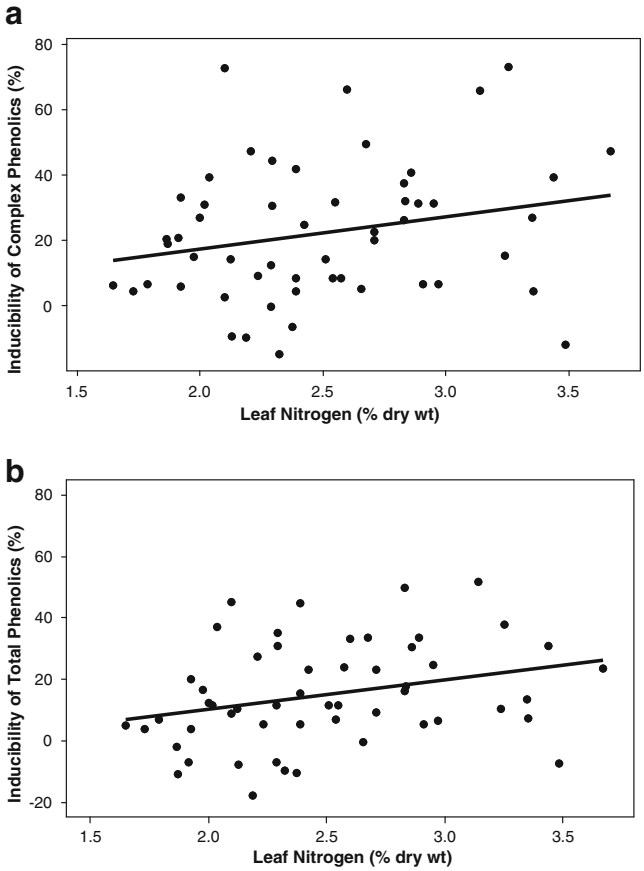


Fig. 4 Relationship between nitrogen content of *Brassica rapa* leaves and inducibility (% change during 24 hr after damage) of (**a**) complex phenolics (R=0.24, P=0.084) and (**b**) total phenolics for plants grown at both CO_2 levels (R=0.29, P=0.033)

of carbon-based phenolics would also be higher (Gayler et al., 2008). Indeed, constitutive levels increased significantly under elevated CO_2 for complex (17%) and total phenolics (14%). These increases are larger than the mean increases of 1% and 5% reported for phenolics in shrubs and forbs, respectively, similar to the 10–14% increases in trees, but smaller than the 29% increase reported for tannins in dicots (Peñuelas et al., 1997; Stiling and Cornelissen, 2007). It is likely that increased constitutive phenolic levels observed in this study will result in more effective defense in *B. rapa*, since phenolic compounds can reduce growth (Ossipov et al., 2001; Haukioja et al., 2002) and survivorship (Karowe, 1989; Haviola et al., 2007) of insect herbivores.

Given the observed increase in constitutive phenolics in *B. rapa* under elevated CO_2, greater inducibility of phenolics under elevated CO_2 might also be expected. However, we observed the opposite: phenolics were less inducible under elevated CO_2. Induction of simple phenolics occurred only for plants grown under ambient CO_2. In addition, although induction of complex and total phenolics occurred under both ambient and elevated CO_2, the damage-induced increases were 64% and 75% smaller, respectively, under elevated CO_2. Therefore, the results of this study suggest that rising atmospheric CO_2 will decrease the ability of *B. rapa* to respond to herbivore damage by rapidly increasing levels of phenolic defenses.

We hypothesize that, in *B. rapa*, decreased leaf nitrogen levels under elevated CO_2 constrain the rapid mobilization of nitrogen required to produce additional jasmonic acid elicitor, mRNA and enzymes required for induction of phenolics, but do not constrain the long-term accumulation of higher levels of constitutive phenolics. In the present study, constitutive phenolic levels under elevated CO_2 were positively correlated with leaf C:N ratio, as would be expected if carbon, rather than nitrogen, availability was the primary determinant of constitutive phenolic levels. In contrast, inducibility of both simple and complex phenolics were positively correlated with leaf N (and negatively correlated with leaf C:N ratio), as would be expected if nitrogen availability were an important determinant of inducibility. Consistent with this hypothesis, Lou and Baldwin (2004) observed that, when grown under low nitrogen, tobacco plants showed decreased induction of chemical defenses, including the phenolics rutin and chlorogenic acid. Moreover, fewer than half of the genes upregulated by herbivore attack under high nitrogen were upregulated under low nitrogen.

Despite the importance of induction to plant defense strategy (Karban and Baldwin, 1997), surprisingly few studies have addressed the effect of elevated CO_2 on the inducibility of chemical defenses in response to herbivory. Most existing studies have not detected an effect of elevated CO_2 on inducibility. For instance, growth under elevated CO_2 did not affect the inducibility of phenolic glycosides or condensed tannin in aspen (Lindroth and Kinney, 1998; Roth et

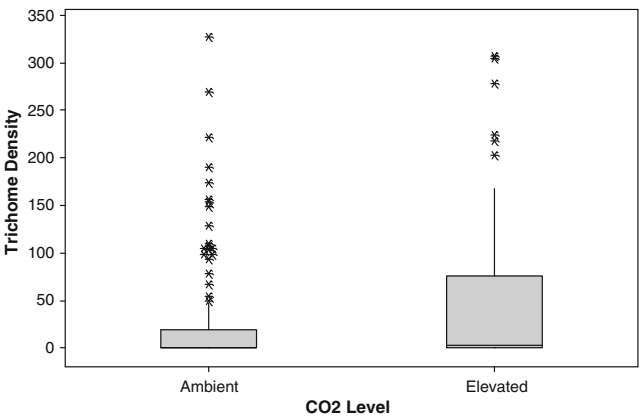


Fig. 5 Number of trichomes per cm^2 of leaf tissue (mean ± SE) in *Brassica rapa* grown under ambient and elevated CO_2[a]. [a]*Shaded* areas include the first through third quartiles, *horizontal lines* indicate medians (median = 0 for ambient CO_2), *vertical lines* indicate the range, and *asterisks* indicate outliers. Sample size is 115 at each CO_2 level. Trichome density was significantly higher at elevated CO_2 (P=0.015)

al., 1998), tannins in myrtle oak (Rossi et al., 2004), or saponins in alfalfa and triterpenoid aldehydes and gossypol in cotton (Agrell et al., 2004). The only two studies to detect a significant effect report contrasting responses: glucosinolates in *Arabidopsis thaliana* induced only under elevated CO_2 (Bidart-Bouzat et al., 2005), while elevated CO_2 reduced induction of soybean cysteine protease inhibitors by 31% (Zavala et al., 2008).

It is possible that the methodology used in this study enhanced the ability to detect an effect of elevated CO_2 on inducibility. In all previous studies of which we are aware, constitutive and induced levels were compared between separate leaves (Rossi et al., 2004) or separate plants (Lindroth and Kinney, 1998; Roth et al., 1998; Agrell et al., 2004; Bidart-Bouzat et al., 2005; O'Neill et al., 2010). These approaches require the assumption that constitutive defense levels are similar between leaves or plants; to the extent that this assumption is violated (Boege and Marquis, 2005), power to detect a difference in inducibility will be reduced. In contrast, the present study compared constitutive and induced levels within a single leaf, and thus only requires the more reasonable assumption that constitutive defense levels were similar between opposite halves of the same leaf.

It should be noted that mechanical damage such as that used in this study can result in patterns of induction that differ from those produced by herbivore damage (Reymond et al., 2000), since herbivore saliva contains elicitors (Musser et al., 2006). However, in general, there is broad similarity in the transcriptional responses to mechanical and herbivore damage (reviewed in Howe and Jander, 2008). For instance, in Sitka spruce, transcriptome changes in response to mechanical wounding overlapped considerably with those in response to beetle and caterpillar herbivory (Ralph et al., 2006). Moreover, differences in response to mechanical vs. herbivore damage may be greater for volatile compounds than for the within-leaf phenolic defenses we assayed here (Howe and Jander 2008). Nonetheless, given that we applied neither herbivore oral secretions nor jasmonic acid to the site of mechanical damage, we cannot say with certainty whether the patterns of defense induction we observed would occur in response to actual herbivory.

We also observed a significant 57% increase in trichome density for *B. rapa* grown under elevated CO_2, and a 41% increase in the proportion of leaf discs that contained at least one trichome. It is likely that an increase of 57% in trichome density would result in enhanced antiherbivore defense, since slower growth and higher mortality of the two most important crucifer specialists, *Pieris rapae* and *Phyllotreta cruciferae*, were associated with a 43% increase in trichome density in a related species, *Brassica nigra* (Traw and Dawson, 2002). Higher trichome density may also increase *B. rapa* resistance to pathogens (Lake and Wade, 2009).

We are not aware of any other study demonstrating an increase in trichome density that is clearly attributable to elevated CO_2. Paoletti et al. (2007) report increased trichome density in *Quercus ilex* growing near a natural CO_2 spring, but plants also were exposed to elevated levels of H_2S and SO_2. In contrast to our results, trichome density decreased under elevated CO_2 in *Arabidopsis thaliana* (Bidart-Bouzat et al., 2005; Lake and Wade, 2009), and did not change in birch (Riikonen et al., 2010), milkweed (Vannette and Hunter, 2011), or round headed bush clover (Lau et al., 2008). Given this variation in response, trichomes should remain a high priority subject for elevated CO_2 studies.

In summary, our results suggest that *Brassica rapa* will possess increased constitutive levels of both phenolic compounds and trichomes as atmospheric CO_2 continues to rise, which are likely to increase resistance to herbivores, but that phenolics will be less responsive to herbivore attack, which is likely to decrease resistance to herbivores. Whether these changes together will be advantageous, neutral, or disadvantageous will depend on the relative importance of chemical vs. mechanical defenses and, particularly, of constitutive vs. induced chemical defenses for *B. rapa*.

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from *Tectona grandis* and the assessment of their activity would help to explain the success of this tree culture when it is used in agroforestry. At present, no natural compound with high phytotoxic activity has been isolated from teak in a great enough amount that could explain that activity.

In addition, this species has attracted other scientific interest. The consideration of teak in Natural Products research is based on its ethnobotanical uses, the biological activity shown by its extracts, and the durability of its wood (e.g., Ahuwalia and Seshadri, 1957; Rudman, 1960; Joshi et al., 1977; Marwani et al., 1997; Khan and Mlungwana, 1999; Macías et al., 2008, 2010; Sumthong et al., 2008; Naira and Kaverkar, 2010; Singh et al., 2010; Shukla et al., 2010). In addition to the value of the cultivation of *T. grandis* as source of wood and the extension of forest areas, it also has become an efficient tool for pest control in the context of a sustainable agriculture.

Previously, we reported the isolation of apocarotenoids and terpenoids from bioactive extracts from leaves of *Tectona grandis* (Macías et al., 2008, 2010). As a continuation of our systematic phytochemical and allelopathic study of this species, we describe here the isolation, structural elucidation of two new quinones (one isoprenoid quinone and one anthraquinone) shown in Fig. 1, and the phytotoxic activity of the most abundant one. Naphthoquinones, anthraquinones, and isoprenoid quinones are abundant metabolites in teak. Among naphthoquinones isolated from teak, lapachol (Joshi et al., 1977) and structurally related metabolites (Khan and Mlungwana, 1999) have been reported. Anthraquinones isolated from this species usually have a carbonyl group at positions 9 and 10, and the simplest one is tectoquinone (Ahuwalia and Seshadri, 1957; Agarwal et al., 1965). This compound has shown activity as a termite repellent (Rudman, 1960). These compounds were isolated mainly from the bark. This is the first time that one isoprenoid quinone is described from leaves of teak.

Methods and Materials

General IR spectra (KBr) were recorded on a Perkin-Elmer FT-IR Spectrum 1000 or a Matton 5020 spectrophotometer.

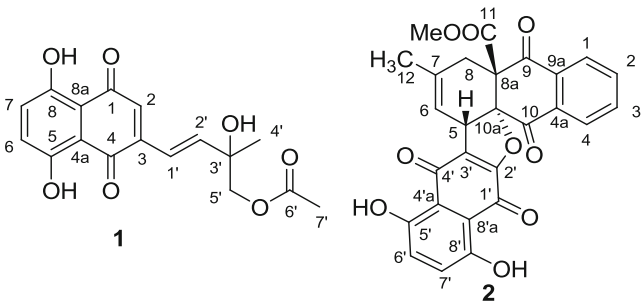


Fig. 1 Quinones isolated from *Tectona grandis*. 1 = *Naphthotectone*, 2 = *Anthratectone*

NMR spectra were run on Varian INOVA-400 and Varian INOVA 600 spectrometers. Chemical shifts are given in ppm with respect to residual ^{1}H signals of $CHCl_3$-d_1 (δ 7.25), and ^{13}C signals are referenced to the solvent signal (δ 77.0). Optical rotations were determined using a Perkin-Elmer model 241 polarimeter (on the sodium D line). HRMS were obtained on a VG AUTOESPEC mass spectrometer (70 eV). HPLC was carried out on a Merck-Hitachi instrument, with RI detection, using two different Merck LiChrospher columns: SI 60 (5 μm, 250×4 mm) and SI 60 (10 μm, 250×10 mm).

Plant Material Leaves of *Tectona grandis* L. f. were collected between the months of February and March (2003) in Ciudad de La Habana and were identified by MsC. Lutgarda González. Voucher specimens (80613) were deposited at the Jardín Botánico de Cuba.

Extraction and Isolation Dried leaves of *Tectona grandis* (5.0 kg) were extracted with water (35 L) for 24 h at room temperature in the dark. The aqueous solution was extracted with CH_2Cl_2 and then with EtOAc at room temperature. The residual vegetal material was extracted with methylene chloride (DCM) and then with methanol. DCM extract was one of the most active ones. Details of the extraction procedure and the bioassays on the extract have been described previously (Macías et al., 2008).

The DCM (120 g) extract was chromatographed on silica gel (2.0 kg) using hexane/ethyl acetate mixtures of increasing polarity to yield eight fractions: A_2–H_2.

Further purification of fraction $C_2$2 ($CHCl_3$, 0.906 g) by CC using hexane/chloroform and chloroform/acetone mixtures on silica gel and hexane/chloroform/methanol (3:1:1) on Sephadex LH-20 and silica gel HPLC (hexane/chloroform, 1:1) afforded 1.0 mg of compound **2** (Fig. 1).

Details of the separation procedure on the fraction D_2 (hexane/EtOAc, 3:3, 7.46 g) have been described previously (Macias et al., 2010). Finally, purification of the fractions $D_2$2B, $D_2$2C, and $D_2$3B using hexane/chloroform and chloroform/acetone mixtures afforded 60 mg of compound **1** (Fig. 1).

Fraction E_2 (hexane/EtOAc, 1:1, 12.0 g) was subjected to CC on Sephadex LH-20 using hexane/chloroform/methanol (2:1:1) to afford 7 fractions ($E_2$1–$E_2$7). Fraction $E_2$4 (2.24 g) was subjected to CC on silica gel using mixtures of hexane/acetone. Fractions were combined into 8 fractions ($E_2$4A–$E_2$4H). Fraction $E_2$4C (0.300 g) was chromatographed on silica gel CC using mixtures of hexane/chloroform and chloroform/acetone. Less polar fractions afforded 8 mg of compound **1**.

*Naphthotectone (**1**)* (Fig. 1): Amorphous red solid; $[\alpha]_D^{25}$= +115.4 (*c* 0.10, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}; 3462 (OH),

1738 (C=O), 1608 (C=C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 12.67 (1H, s, OH), 12.47 (1H, s, OH), 7.16 (1H, d, J=0.4 Hz, H-2), 7.05 (2H, s, H-6, H-7), 6.90 (1H, d, J=16.2 Hz, H-1′), 6.68 (1H, d, J=16.2 Hz, H-2′), 4.20 (1H, d, J=16.2 Hz, H-5b′), 4.06 (1H, d, J=16.2 Hz, H-5a′), 2.10 (3H, s, H-7′), 1.41 (3H, s, H-4′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.5 (C, C-1), 178.9 (C, C-4), 171.0 (C, C-6′), 166.3 (C, C-5), 165.5 (C, C-8), 142.9 (C, C-3), 142.5 (CH, C-2′), 132.5 (CH, C-6), 132.1 (CH, C-7), 130.4 (CH, C-2), 121.1 (CH, C-1′), 112.0 (C, C-4a), 111.8 (C, C-8a), 72.8 (C, C-3′), 70.6 (CH_2, C-5′), 24.8 (CH_3, C-4′), 20.8 (CH_3, C-7′); HREIMS m/z 332.0892 (calc. for $C_{17}H_{16}O_7$ m/z 332.0896).

*Anthratectone (***2***)* (Fig. 1): Orange oil; $[\alpha]_D^{25}$=+1.7 (c 0.01, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}; 3418 (OH), 1731, 1715, 1697 (C=O). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 12.61 (1H, s, OH), 12.02 (1H, s, OH), 8.28 (1H, d, J=7.6 Hz, H-1), 7.96 (1H, d, J=7.6 Hz, H-4), 7.82 (1H, ddd, J=7.6 Hz, J=7.6 Hz, J=1.6 Hz, H-2), 7.70 (1H, ddd, J=7.6 Hz, J=7.6 Hz, J=0.8 Hz, H-3), 7.22 (1H, d, J=9.2 Hz, H-6′), 7.13 (1H, d, J=9.2 Hz, H-7′), 5.91 (1H, dq, J=6.7 Hz, J=1.5 Hz, H-6), 4.82 (1H, d, J=6.7 Hz, H-5), 3.66 (3H, s, OCH_3), 3.54 (1H, brd, J=20 Hz, H-8b), 2.70 (1H, dd, J=20 Hz, J=0.6 Hz, H-8a), 1.72 (3H, s, H-12); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 188.2 (C, C-10), 187.9 (C, C-1′), 187.4 (C, C-9), 185.4 (C, C-4′), 166.6 (C, C-11), 158.3 (C, C-8′), 157.3 (C, C-5′), 151.8 (C, C-2′), 136.3 (C, C-7), 135.8 (CH, C-2), 134.6 (CH, C-3), 133.3 (C, C-9a), 131.8 (C, C-4a), 130.5 (CH, C-7′), 128.7 (CH, C-6′), 128.4 (CH, C-1), 127.7 (CH, C-4), 126.4 (C, C-3′), 122.2 (CH, C-6), 111.6 (C, C-8a′), 110.6 (C, C-4a′), 81.3 (C, C-10a), 63.8 (C, C-8a), 53.8 (OCH_3), 38.7 (CH_2, C-8), 29.1 (CH, C-5), 22.1 (CH_3, C-12); HREIMS m/z 486.0961 (calc. for $C_{27}H_{18}O_9$ m/z 486.0951).

Coleoptiles Bioassay Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in 15 cm diam Petri dishes moistened with water and grown in the dark at 22±1ºC for 3 d (Hancock et al., 1964). Roots and caryopses were removed from the shoots. The latter were placed in a Van der Weij guillotine, and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for bioassays. All manipulations were performed under a green safelight (Nitsch and Nitsch, 1956). Compounds were predissolved in DMSO and diluted to the final bioassay concentration with a maximum of 0.1% DMSO. Parallel controls were also run.

Fractions or pure compounds to be assayed for biological activity were added to test tubes. Phosphate-citrate buffer (2 ml) containing 2% sucrose (Nitsch and Nitsch, 1956) at pH 5.6 was added to each test tube. Five coleoptiles were placed in each test tube (3 tubes per dilution) and the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 22ºC in the dark. Coleoptiles were measured by digitalization of their images. Data were statistically analyzed using Welch's test (Martín Andrés and Luna del Castillo, 1990). Data are presented as percentage differences from control. Thus, zero represents the control; positive values represent stimulation of the studied parameter, and negative values represent inhibition.

Phytotoxicity Bioassay The selection of target plants was based on an optimization process carried out by us in our search for a standard phytotoxicity bioassay (Macías et al., 2000). Several Standard Target Species (STS) were proposed, including monocots *Triticum aestivum* L. (wheat) and *Allium cepa* L. (onion), and dicots *Lycopersicon esculentum* Will. (tomato), *Lepidium sativum* L. (cress), and *Lactuca sativa* L. (lettuce), which were assayed for this study.

Bioassays were conducted using Petri dishes (50 mm diam) with one sheet of Whatman No.1 filter paper as a support. Germination and growth were conducted in aqueous solutions at controlled pH by using 10^{-2} M 2-[*N*-morpholino]ethanesulfonic acid (MES) and 1 M NaOH (pH 6.0). The compound to be assayed was dissolved in DMSO (0.1, 0.02, 0.01, and 0.002 M), and these solutions were diluted with buffer (5 µl DMSO solution/ml buffer) so that test concentrations for each compound (10^{-3}, $5{\cdot}10^{-4}$, 10^{-4}, $5{\cdot}10^{-5}$, and 10^{-5} M) were achieved. This procedure facilitated the solubility of the assayed compound. The number of seeds in each Petri dish depended on the seed size. 20 seeds were used for tomato, lettuce, cress, and onion. Treatment, control, or internal reference solution (1 ml) was added to each Petri dish. A similar procedure was used for wheat in 90 mm diam Petri dishes with 10 seeds. Four replicates were used for tomato, cress, onion, and lettuce (80 seeds) whereas ten replicates (100 seeds) were used for wheat.

After adding seeds and aqueous solutions, Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds were further incubated at 25ºC in a Memmert ICE 700 controlled environment growth chamber in the dark. Bioassays took 4 d for cress, 5 d for lettuce, tomato, and wheat, and 7 d for onion. After growth, plants were frozen at −10ºC for 24 h to avoid subsequent growth during the measurement process.

The commercial herbicide Logran®, which original formulation from Syngenta is a combination of *N*-(1,1-dimethylethyl)-*N*′-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (Terbutryn, 59.4%) and 2-(2-chloroethoxy)-*N*-{[(4-methoxy-6-methyl-1,3,5-triazin- 2yl)amino]carbonyl}benzene-sulfonamide (Triasulfuron, 0.6%), was used as an internal reference according to a comparison study reported previously (Macías et al., 2000). This reference was used at the same concentrations (10^{-3}, $5{\cdot}10^{-4}$, 10^{-4}, $5{\cdot}10^{-5}$, and 10^{-5} M) and under the same conditions as reported

previously. Control samples (buffered aqueous solutions with DMSO and without any test compound) were used for all of the plant species assayed.

Evaluated parameters (germination rate, root length, and shoot length) were recorded using a Fitomed© system (Castellano et al., 2001), which allowed automatic data acquisition and statistical analysis using its associated software. Data were analyzed statistically using Welch's test, with significance fixed at 0.01 and 0.05. Results are presented as percentage differences from the control. Zero represents control, positive values represent stimulation, and negative values represent inhibition.

IC_{50} values were obtained after adjusting phytotoxicity data to concentration (logarithmic scale), to a sigmoidal dose-response curve, defined by the equation:

$$Y = Y_{\min} + \frac{Y_{\max} - Y_{\min}}{1 + 10^{\log EC50 - X}}$$

Where X indicates the logarithm of concentration, Y indicates the response (phytotoxicity), and $Y_{\max}$ and $Y_{\min}$ are the maximum and minimum values of the response, respectively. Goodness of fit is described by the determination coefficient (r^2). The adjustment and the r^2 value were obtained using GraphPad Prism® software v. 4.00.

Results and Discussion

The study of the allelopathic potential of *Tectona grandis* and its possible use as a source of natural herbicide models was continued with the phytochemical study of DCM extract obtained by direct maceration of the dry leaves. This extract was selected on the basis of the bioactivity levels shown in the wheat coleoptiles bioassay (Macías et al., 2008).

*Naphthotectone (***1.***)* Compound **1** is the major compound of this extract. We estimate that the percentage of **1** is aproximately 0.2% by weight of the original extract. It was isolated as an amorphous red solid. The molecular formula was determined to be $C_{17}H_{16}O_7$ from the molecular ion peak at *m*/*z* 332.0892 (Calcd. 332.0896) in the HR-EIMS. The IR absorptions at 3462, 1738, 1608, and signals in the ^{1}H NMR spectrum at δ 7.16 (1H, d, J = 0.4 Hz, H-2), 7.05 (2H, s, H-6 and H-7), 2.10 (3H, s, $C\underline{H}_3CO$), and two downfield singlets (δ 12.47 and 12.67) indicate the presence of a naphthoquinone derivative with a hydroxy group *peri* to either carbonyl function, and an acetylated alkyl substitution. This proposed formula is further supported by the similarity of these spectroscopic data to those reported for other analogous compounds such as acetylshikonin (Ahn et al., 1995). In the ^{13}C NMR spectrum, the carbonyl carbons resonated at δ_C 179.5 (C-1), 178.9 (C-4), and 171.0 ($CH_3–\underline{C}O$). Besides the carbonyl carbons, the ^{13}C NMR spectrum displayed 14 signals, 2×CH_3, 1×CH_2, 5×CH and 6×C, which were assigned with the aid of g–HSQC and g–HMBC spectra.

Besides the acetyl signal, the ^{1}H NMR spectrum shows 5 signals corresponding to the alkyl side chain located at C-3: a singlet at δ 1.41 (3H, s, H-4′) corresponding to a methyl group attached at to oxygenated carbon; an AB system at δ 4.06 (1H, *d*, J=16.2 Hz, H-5a′) and 4.20 (1H, *d*, J=16.2 Hz, H-5b′), which is consistent with an acetylated methylene; and two vinyl protons at δ 6.68 (1H, *d*, J=16.2 Hz, H-2′) and 6.90 (1H, *d*, J=16.2 Hz, H-1′) whose coupling constants imply an *E* geometry. These data, together with the correlations observed in the g-HMBC spectrum (Fig. 2), allow us to propose structure **1** for this compound. This structure has not been described previously in the literature and we have named this compound naphthotectone.

*Anthratectone (***2***)* Compound **2** was isolated as an orange oil. The IR absorptions at 1731, 1715, 1697, and two downfield singlets (δ 12.61 and 12.02) in the ^{1}H NMR spectrum suggest the presence of a quinone with a hydroxy group *peri* to two of the carbonyl groups. The molecular formula was determined to be $C_{27}H_{18}O_9$ from the molecular ion peak at *m*/*z* 486.0961 (Calcd. 486.0951) in the HR-EIMS.

The ^{1}H NMR spectrum showed two singlets at δ 1.72 (3H, bs) and 3.66 (3H, s), and these are assigned to a methyl group attached to a double bond and a methoxyl group, respectively. Additionally, we observed two geminal protons at δ 3.54 (1H, d, J=20 Hz, H-8b) and δ 2.70 (1H, d, J=20 Hz, H-8a) and two coupled signals δ 4.82 (1H, d, J=6.7 Hz, H-5) and δ 5.91 (1H, dq, J=6.7, J=1.5 Hz, H-6).

The presence of an isoprenyl chain can be inferred from the analysis of the ^{1}H NMR-2D-COSY spectrum. Thus, the signal at δ 1.72 (3H, bs) showed a small coupling with the geminal system at δ 3.54 (H-8b) and δ 2.70 (H-8a), and signals at δ 4.82 (H-5) and δ 5.91 (H-6).

In the aromatic region of the ^{1}H NMR spectrum, two groups of coupled signals can be distinguished. The first one [δ 7.70 (1H, ddd, J=7.6 Hz, J=7.6 Hz, J=0.8 Hz, H-3), δ 7.82 (1H, ddd, J=7.6 Hz, J=7.6 Hz, J=1.6 Hz, H-2), δ 7.96 (1H, d, J=7.6 Hz, H-4) and δ 8.28 (1H, dd, J=7.6 Hz, J=7.6 Hz, J=0.8 Hz, H-1)] suggests the presence of a 1,4-anthraquinone system. The second [(δ 7.13 (1H, d,

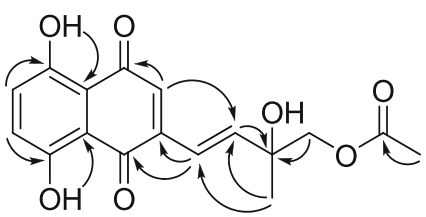


Fig. 2 Heteronuclear Multiple-Bond Connectivity (HMBC) correlations observed for compound **1**

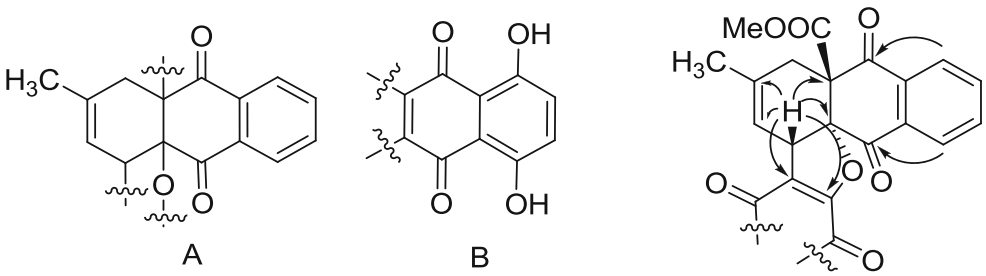


Fig. 3 Selected Heteronuclear Multiple-Bond Connectivity (HMBC) correlations observed for compound **2**

J=9.2 Hz, H-3) and δ 7.22 (1H, d, J=9.2 Hz, H-2)], together with signals at δ 12.61 and 12.02, are typical of a 5,8-dihydroxy-1,4-naphthoquinone.

The ^{13}C NMR spectrum contained 27 signals: 2 methyls, 1 methylene, 8 methynes and 16 quaternary carbons, according to an HSQC experiment. Two of the quaternary carbons are sp^3, at δ 63.8 and 81.3, and this implies that they are attached to withdrawing groups. Five signals appear to be due to carbonyl groups at δ 188.2, 187.9, 187.4, 185.4, and 166.6. The latter signal was correlated with the methoxyl group (δ 3.66) in the HMBC experiment. This indicates the presence of a methyl ester and two quinone units. Other correlations confirm the presence of an isoprenoid chain: H-5 (δ 4.82) with C-10a (δ 81.3) and C-10 (δ 188.2) as well as H-8b (δ 3.54) and H-8a (δ 2.70) with C-8a (δ 63.8) and C-9 (δ 187.4). These correlations led us to propose partial structure A (Fig. 3), with the isoprenoid chain attached to positions 8a and 10b of the 1,4-naphthoquinone unit. Correlations between H-7′ (δ 7.13) and H-6′ (δ 7.22) with C-5′ (δ 157.3) and C-8′ (δ 158.3) establish partial structure B as a 5,8-hydroxy-1,4-naphthoquinone (Fig. 3).

The linkage between these two sub-structures (Fig. 3) was established on the basis of the HMBC correlations between H-5 (δ 4.82) and the carbons C-8a (δ 63.8), C-10a (δ 81.3), C-3′ (δ 126.4), C-2′ (δ 151.8), C-4′ (δ 185.4), C-1′ (δ 187.9), and C-2′ (δ 151.8); as well as the chemical shift of C-2′ and the molecular formula.

The stereochemistries at C-8a and C-5 were deduced from the NOE effects observed on H-5 and H-12 on irradiation of the methoxyl group. Only an alpha configuration for the oxygen at the furan ring is possible with this stereochemistry. This structure has not been described previously and we have named this compound anthratectone.

The biosynthesis of this compound could be explained by the union of the two naphthoquinones I and II (Fig. 4) by a *syn-endo* Diels–Alder reaction, which explains the stereochemistry observed, and the subsequent formation of the furan ring by intramolecular cyclization.

Bioassays The etiolated wheat coleoptiles bioassay is a rapid test that is sensitive to a wide range of bioactive substances (Cutler, 1984; Cutler et al., 2000; Jacyno and Cutler, 1993). A very small amount of compound **2** was isolated and, therefore, only compound **1** could be evaluated. The highest concentration tested was 10^{-3} M. The activity was inhibitory at all concentrations tested and was greater than −80% at the most concentrated, with the activity retained upon dilution: (−81%, 10^{-3} M; −77%, $5{\cdot}10^{-4}$ M; −55%, 10^{-4} M; −46%, $5{\cdot}10^{-5}$ M; −25%, 10^{-5} M). The IC_{50} values were calculated using the sigmoidal dose-response model,

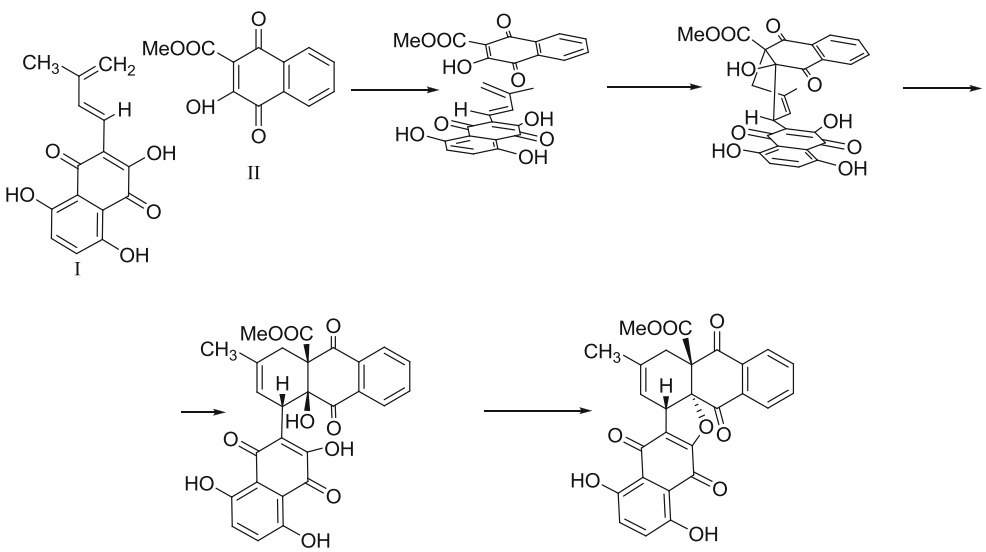


Fig. 4 Biosynthetic proposal for compound **2**

affording a value of 0.138 mM (R^2=0.974). These values are similar to those obtained for the commercial herbicide Logran® (−90%, 10^{-3} M; −87%, $5{\cdot}10^{-4}$ M; −75%, 10^{-4} M; −54%, $5{\cdot}10^{-5}$ M; −18%, 10^{-5} M), used as positive control. If we compare this result with the activities of other compounds isolated and assessed by our research group from teak, only solidagonal acid (IC_{50}=0.078 mM) (Macias et al., 2010) is sligthly more active than naphthotectone. Nevertheless, the ecological and defensive relevance of the activity of this compound should be higher, as it is much more abundant than solidagonal acid in the extract.

Phytotoxicity Bioassay Results The concentrations tested in the phytotoxicity assay were identical to those in the previous bioassay. The standard target species (STS) were *Lactuca sativa* L. (lettuce), *Lycopersicum esculentum* Will. (tomato), *Lepidium sativum* L. (cress), and *Allium cepa* L. (onion) (Fig. 5). Regarding the dicotyledonous species, the compound under investigation did not cause significant effects on germination or growth of *Lepidium sativum*.

The behavior of **1** in the germination and growth of *Lactuca sativa* was inhibitory in almost all cases. The highest inhibitory effect was observed on germination, where compound **1** showed activity values similar to those of the herbicide Logran® at the highest concentrations (10^{-3} M, −89%; $5{\cdot}10^{-4}$ M, −89%; 10^{-4} M, −66%) and higher values at lower concentrations ($5{\cdot}10^{-5}$ M, −55%; 10^{-5} M, −32%). The activities on root and shoot growth were more persistent with dilution than those of Logran®. Thus, we highlight the inhibition of root growth at 10^{-3} M (−62%) and $5{\cdot}10^{-4}$ M (−49%).

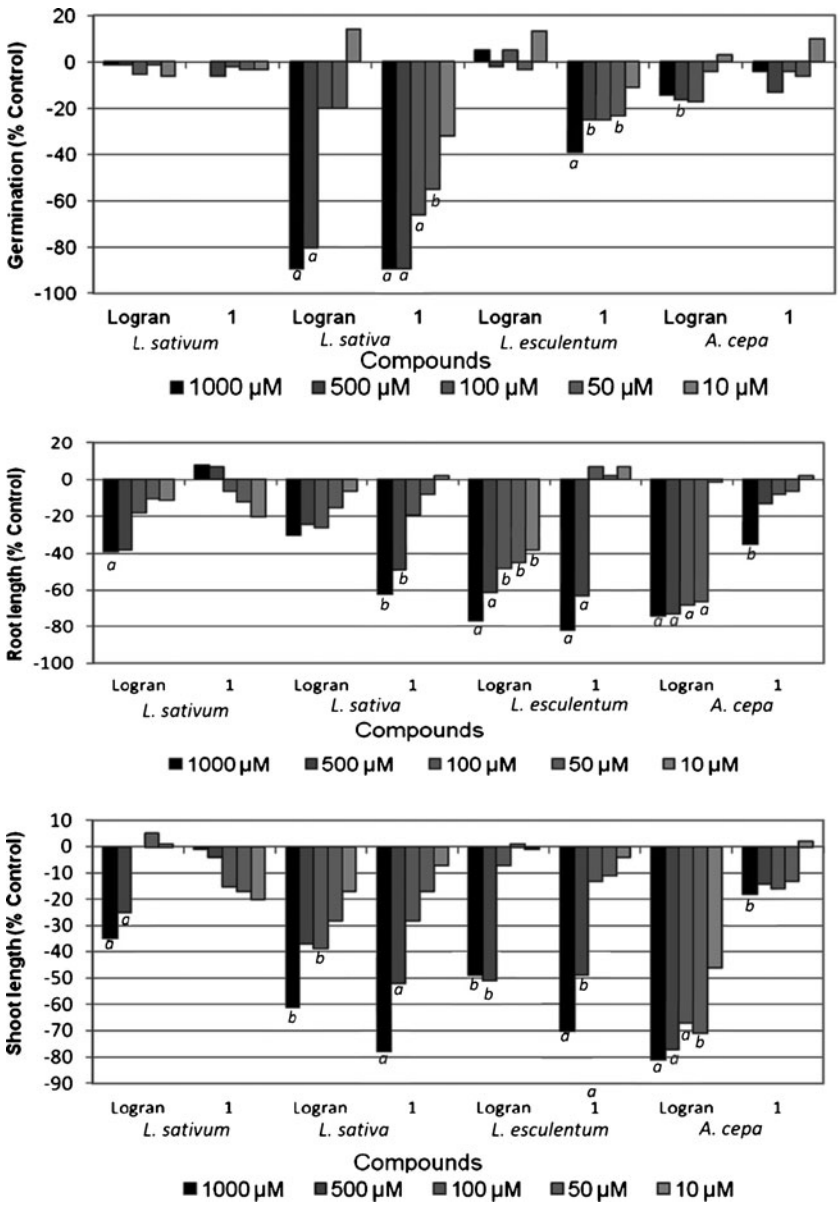


Fig. 5 Effects of the commercial herbicide Logran and compound **1** on the growth of standard target species. Values are expressed as percentages from the control and are not significantly different (P>0.05 for the Mann–Whitney test). [a]Values significantly different with P< 0.01. [b]Values significantly different with 0.01<P<0.05

With regard to the third dicotyledonous species, *Lycopersicum esculentum*, the effects on germination were higher (−40% at 10^{-3} M) than those of Logran®. Root growth of tomato was more inhibited, with values of −82% and −63% at 10^{-3} M and $5{\cdot}10^{-4}$ M, respectively. Shoots were less affected, but once again the effects were higher than those obtained with Logran®.

In the monocotyledon species *Allium cepa*, the most affected parameter was root growth in terms of inhibitory activities. The effects on germination and shoot development were not significant.

Compound **1** presented high activities in both bioassays. This finding could be of importance from the ecological point of view since the new compound identified is one of the most abundant metabolites in *Tectona grandis* and presents a high phytotoxic activity. Natural naphthoquinones and anthraquinones are compounds with a broad spectrum of biological activities (Abad and Bermejo, 2005) against microbes, trypanosomes, viruses, and cancer cells, and it has been demonstrated that some quinones can inhibit enzymes that triggers apoptosis (Silva et al., 2003).

Regarding their allelopathic activity, they were early described as phytotoxic agents, although currently the number of these compounds that have shown activity is comparatively low. Thus, one of the first examples of an allelochemical to be described was juglone (5-hydroxy-1,4-naphthoquinone), isolated from black walnut tree (*Juglans nigra* L.)(Davis, 1928; Shibu, 2002). Another well-known example is sorgoleone and its related compounds (Kagan et al., 2003) that are the responsible for the allelopathic effect of sorghum (Netzly and Butler, 1986). Both juglone and sorgoleone are highly phytotoxic and their modes of action have been studied (Hejl et al., 1993; Dayan et al., 2010). So, compound **1** may be one of the major compounds responsible for the allelopathic activity shown by teak as well as being involved in other defense mechanisms. Explanation of this activity is a major goal of our work. This is the initial study, and it is necessary for further experiments to detect this compound in soil, and in the target species. The fate of this compound should be studied, as well as the bioactivity of the possible degradation products.

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quantitatively within species, between sexes, populations, individuals (Soini et al., 2007; Mardon et al., 2010; Whittaker et al., 2010), and age classes (Shaw et al., 2011).

In seasonally reproducing species, many physiological and behavioral changes occur during the transition from non-breeding to breeding condition. Many of these changes are mediated by steroid hormones induced by changes in photoperiod (Farner and Wingfield, 1980). Our study species, the dark-eyed junco (*Junco hyemalis*), is a North American passerine bird that breeds in the summer months. In juncos, like many seasonally breeding birds, circulating testosterone (T) levels increase sharply early in the breeding season, drop when the female lays eggs, and stay low while both parents care for the offspring (Ketterson et al., 2001, 2005). This pattern is observed in both sexes, although T levels typically are higher in males than females at most or all stages of breeding (Ketterson et al., 2001, 2005). Throughout the breeding season, males respond to an injection of gonadotropin-releasing hormone (a "GnRH challenge") with a short-term increase in T (Jawor et al., 2006). The only time that females respond this way is during the 7 days before egg laying, when they are depositing yolk in eggs (Jawor et al., 2007).

In juncos, concentrations of preen oil volatile and semi-volatile compounds are higher in captive birds held in long-day, compared to birds in short-day photoperiods, especially for the linear 1-alkanols, 1-undecanol through 1-hexadecanol, the methyl ketones, 2-undecanone through 2-pentadecanone, and the carboxylic acids, dodecanoic acid, tetradecanoic acid, and hexadecanoic acid (Soini et al., 2007). For convenience, we henceforth will refer to both volatile and semivolatile compounds as "volatile compounds," but acknowledge that the heavier compounds with lower vapor pressures, such as hexadecanoic acid, are considered "semivolatile." Males have significantly higher proportions of two methyl ketones, 2-tridecanone and 2-pentadecanone, in preen oil, while females have a blend with higher proportions of 1-undecanol and the three carboxylic acids above (Whittaker et al., 2010). The function of these compounds is not yet clear, but they appear capable of transmitting information about identity that could be used in mate choice (Mardon et al., 2010; Whittaker et al., 2010). Juncos in breeding condition detect these odors and exhibit preferences based on sex and individual characteristics of the bird that produced the preen oil (Whittaker et al., 2011).

This relationship among volatile compound concentrations, sex, and reproductive condition suggests that changes in preen oil concentration may be linked to hormone levels, even though the role of hormones in mediating changes in avian chemosignal production is not well understood. The uropygial gland contains androgen receptors (Daniel et al., 1977; Shanbhag and Sharp, 1996), and treatment with exogenous T increased rates of protein synthesis in the uropygial gland (Maiti and Ghosh, 1972; Amet et al., 1986). The similarity of the avian uropygial gland and the testosterone-dependent mammalian preputial gland has been noted previously (Maiti and Ghosh, 1972). The mammalian preputial gland can be the source of pheromones related to reproductive behavior, such as the farnesenes secreted by the mouse preputial gland (Novotny et al., 1990). Certain volatile compounds and proteins produced by the rat preputial gland are stimulated by testosterone (Ponmanickam et al., 2010). These observations suggest that, in birds, T might stimulate production of volatile compounds in the uropygial secretion. However, in the only study to examine this relationship so far, concentrations of volatile carboxylic acids decreased in the preen oil of gray catbirds receiving T treatment (Whelan et al., 2010).

We present two studies designed to elucidate the relationship between variation in steroid hormone levels and concentrations of preen oil volatile compounds. The first studied the increase in preen oil volatile compounds during the early spring, when juncos return to breeding grounds and undergo physiological and behavioral shifts into breeding condition. We asked whether the preen oil of wild juncos would have the same or different composition as captive junco preen oil. We also asked whether the effect of day length on preen oil concentration observed in the laboratory (Soini et al., 2007) was repeated under field conditions, and whether any changes would correlate with the rise in testosterone that is known to occur during early spring.

In the second study, we again used male and female juncos in captivity. The birds were held on short days, and had T levels elevated experimentally in order to test whether T alone, without the influence of increased photoperiod, stimulated production of volatile compounds in preen oil similar to the increases observed during long days. We hypothesized that birds with experimentally elevated T levels would show increases in preen oil volatile compound concentrations. We also asked whether birds with higher levels of T would have greater increases in concentrations of those compounds.

Methods and Materials

Study Species Our study subjects were wild and captive dark-eyed juncos (*Junco hyemalis carolinensis*) from Mountain Lake Biological Station (MLBS), Pembroke, VA, USA (previous studies of chemical communication in juncos have included this subspecies as well as *J. h. thurberi* in southern California and *J. h. aikeni* from South Dakota: Soini et al., 2007; Whittaker et al., 2010, 2011). This population has been the subject of a long-term behavioral and endocrinological study since 1983 (Ketterson et al., 2001, 2005; Nolan et al., 2002). From about April 15 to May 15 of every year, juncos are captured using mist nets and traps to census returning

individuals and to band new birds with USFWS bands. From May 15 to July 15, we intensively search for nests and monitor them from laying to hatching to fledging.

Study 1: Early Season Profiles of Preen Oil Volatile Compound Relative Concentrations in Wild Males and Females We collected preen oil from every bird captured opportunistically during the early season census from April to May 16, 2008. Sex was determined by observation of a brood patch (females) or cloacal protuberance (males), and also by plumage and wing length (Nolan et al., 2002). We collected preen oil by gently rubbing the uropygial gland with a 100 µl glass capillary tube (Drummond Scientific, Broomall, PA, USA), which stimulates the gland to secrete 1–3 mg of preen oil (Whittaker et al., 2010). Most samples were collected soon after capture, to minimize the impact of stress hormones. However, unlike circulating hormone levels in blood, which are known to change very quickly (less than 15 min), preen oil content is not expected to change over such a short time period. We have observed, when we collect preen oil from a bird, it can take more than 24 h before that individual has enough preen oil to sample again (Whittaker, personal observation). Preen oil was stored at −20°C within 10 min of collection and kept frozen until analyzed by gas chromatography–mass spectrometry (GC-MS), as described below. We analyzed the volatile compound content of preen oil from all birds captured on at least three different days over the course of this period, for a total of 16 females and 35 males. To compare the change in compounds over time, we binned the collection dates into 4 weeks, and used week as a categorical variable. We measured GC-MS peak areas for 15 volatile compounds that are known to differ between males and females (Whittaker et al., 2010), and between breeding and non-breeding season (Soini et al., 2007). The data were not normally distributed, so we natural-log transformed peak areas. We used a multivariate analysis of variance (MANOVA) to compare ln-transformed peak areas of each compound across the 4 weeks, and used Fisher's LSD *post-hoc* test to determine when changes occurred. All statistics were two-tailed.

Study 2: Response of Preen Oil Volatile Compounds in Captive Male and Female Juncos Held on Short Days to Experimental Elevation of Testosterone In October 2009, we captured adult juncos at MLBS using mist-nets and transported them to Indiana University, Bloomington, where they were held in an indoor, climate-controlled aviary on a natural light:dark cycle. Birds were given *ad libitum* access to water, millet and sunflower seeds, mealworms, orange slices, and a mixture of puppy chow, hardboiled eggs, and carrots (Whittaker et al., 2010). The temperature was maintained at 60–65°C, and photoperiod was set to match the daylight schedule in Pembroke, VA, until late December 2009, when we fixed the light:dark period at 10 L:14D. During short days, juncos are in non-breeding condition and have low levels of T and low concentrations of preen oil volatile compounds (Ketterson et al., 2005; Soini et al., 2007).

Males and females were randomly assigned to one of three treatment groups: control (C), which received empty implants made from 7 mm Silastic ® tubing (Dow Corning, 1.47 mm i.d., 1.96 mm o.d.); low T, which received implants packed with 2.5 mm (females) or 5 mm (males) of crystalline T (Sigma-Aldrich, Inc.); and high T, which received implants with 5 mm (females) or 10 mm (males) of T (Ketterson et al., 1991, 1992). The high T doses were selected to produce circulating T levels similar to natural peaks observed during the breeding season (Ketterson et al., 1991, 1992, 2005; Clotfelter et al., 2004). The implants were inserted subcutaneously along the left flank after anesthetizing birds with isofluorane.

There were a total of 42 birds in the study: 24 males (8 C, 8 low T, 8 high T) and 18 females (5 C, 6 low T, 7 high T). Sex was confirmed using a molecular sexing technique in which we amplified fragments of the CHD gene on the W and Z chromosomes (Griffiths et al., 1998).

Birds were housed in individual cages across three rooms. Birds were assigned to cages and rooms randomly with respect to treatment, but we maintained similar sex ratios across all three rooms (F:M ≈ 0.8). The birds were able to see and hear each other. A blood sample was taken 1 week before, and 1 week after, implantation to assay for T concentration. We took preen oil samples from the implanted birds 1 week before implantation, and 2 and 4 weeks after implantation (Dec. 28, 2009 and Jan. 11, 2010).

Although randomly assigned to treatment groups, differences in pre-implant preen oil volatile relative concentrations were observed between control males and males assigned to one of the testosterone treatments. Therefore, we restricted our analysis to the change in ln-transformed volatile compound relative concentrations at 2 and 4 weeks after implant, relative to pre-implant values. Because the data were not normally distributed, we compared treatment groups using nonparametric tests (Kruskal-Wallis for comparing three groups, Mann–Whitney *U* test for comparing two groups). All statistics are two-tailed.

Hormone Assays We measured T levels in plasma samples using a commercial enzyme immunoassay (EIA) kit (Assay Designs #901-065). Assay methods are described in detail elsewhere (Clotfelter et al., 2004). The primary change to the manufacturer's protocol is that we added 2000 cpm of tritiated testosterone to the samples in order to calculate extraction efficiencies. We determined testosterone concentrations with a 4-parameter logistic curve-fitting program (Microplate Manager; BioRad Laboratories, Inc.) and corrected them for incomplete recoveries.

Gas Chromatography–Mass Spectrometry (GC-MS) Our GC-MS method has been described previously (Soini et al., 2007; Whittaker et al., 2010). We added an internal standard (8 ng of 7-tridecanone, Sigma-Aldrich Chemical Company, Milwaukee, WI, USA) dissolved in 5 μl of methanol (Baker Analyzed®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA) to samples, and extracted volatile compounds with the Twister® stir bar (10×0.5 mm polydimethylsiloxane, PDMS) for 60 min (Gerstel GmbH, Mülheim an der Ruhr, Germany). After extraction, the stir bar was rinsed with high-purity OmniSolv® water, dried with a paper tissue, and placed in the thermal desorption autosampler tube. Quantitative analysis was performed using an Agilent 6890N gas chromatograph connected to a 5973i MSD mass spectrometer (Agilent Technologies, Inc., Wilmington, DE, USA) with a Thermal Desorption Autosampler and Cooled Injection System (TSDA-CIS 4 from Gerstel). Samples were thermally desorbed in a TDSA automated system, followed by injection into the column with a cooled injection assembly, CIS-4.

All major compounds were identified by comparison to standard substances obtained from Sigma-Aldrich, using mass spectra and retention times (Soini et al., 2007). Peak areas of the identified compounds were used for quantitative comparisons among the groups. Peak areas were integrated either from the TIC (total ion current) profiles or from the post-run selected ion current (SIC) profiles at *m/z* 55 for linear 1-alkanols, *m/z* 58 for 2-ketones and *m/z* 60 for carboxylic acids. Peak areas of the internal standard (7-tridecanone) were integrated from the post-run *m/z* 113 profiles. Peak areas of the compounds of interest were normalized by dividing each peak area by that of the internal standard in corresponding runs, yielding relative concentrations (i.e., relative amounts per 100 μl of preen oil). Relative standard deviation (RSD, a measure of reproducibility) of the internal standard peak area was 13% (N=12).

All work was conducted in compliance with the Bloomington Institutional Animal Care and Use Committee guidelines (BIACUC protocol 09-037).

Results

Study 1: Early-Season Preen Oil Volatile Compound Profiles We confirmed that wild junco preen oil had the same volatile compound composition as captive junco preen oil. We identified the same 19 volatile compounds in wild junco preen oil as found in captive juncos (Soini et al., 2007; Whittaker et al., 2010). Four of the compounds (1-nonanol, 1-heptadecanol, nonanoic acid, and decanoic acid) were below detectable levels in many individuals and were subsequently left out of our statistical analyses; three of these (1-nonanol, nonanoic acid, and decanoic acid) were shown previously to have low intra-individual repeatability (Whittaker et al., 2010).

Relative concentrations of volatile compounds differed by sex (MANOVA, F=16.506, df=15, P<0.001) and by week (F=1.961, df=45, P=0.001), and there was an interaction between sex and week (F=1.634, df=45, P=0.01). In males, we observed a steady increase across the 4 weeks of early spring in nearly all the measured volatile compounds in male preen oil (Fig. 1, left side). For all 15 volatile compounds that we measured in males, their normalized amounts in week 4 were higher than in week 1 (P<0.05, all comparisons). *Post-hoc* tests showed that the only significant week-to-week increase in *n*-alcohols occurred between weeks 2 and 3 (P<0.01, all comparisons). Two methyl ketones, 2-undecanone and 2-dodecanone, showed an increase between weeks 3 and 4 (P<0.05, both comparisons); 2-tridecanone, 2-tetradecanone, and 2-pentadecanone showed an increase, or near-significant increase, only between weeks 2 and 3 (P<0.1). Carboxylic acids did not increase (P>0.05, all comparisons) over the time period.

The pattern in female juncos was different: no change in any compound was observed between weeks 1 and 2, but all volatile compounds had increased by week 3 (P<0.05, all comparisons; Fig. 1, right side, P>0.1).

We also examined the timing of egg laying in this population. The modal date for first egg laid occurred within week 3, both for the whole population and for birds in this study (Fig. 2). Week 3 was also when female volatile compound relative concentrations peaked (Fig. 1). The later, smaller peaks (Fig. 2) in the population are likely the result of re-nesting attempts by birds whose first clutches were destroyed by a predator before we could find them. There were insufficient data to examine individual correlations between egg laying and volatile compounds, but the population results suggest there may be a relationship between the two.

Study 2: Experimental Elevation of T in Captive Birds Held on Short Days Males (N=24) had pre-implant T levels of 0.52±0.15 ng.ml^{-1} (mean±SD), and post-implant T levels of 0.73±0.26 in C males (N=8), 5.11±1.48 in low T males (N=8), and 11.60±3.09 ng.ml^{-1} in high T males (N=8). Females (N=18) had pre-implant T levels of 0.73±0.58 ng.ml^{-1}, and post-implant T levels of 0.60±0.17 in C females (N=5), 5.05±0.84 in low T females (N=6), and 8.11±3.27 ng.ml^{-1} in high T females (N=7). These post-implant T levels were different among the three treatment groups within each sex [ANOVA, F=60.87 (males) and 18.69 (females), df=2, P<0.001 (both comparisons)].

No differences in changes in preen oil volatile compound relative concentrations were observed between the sexes

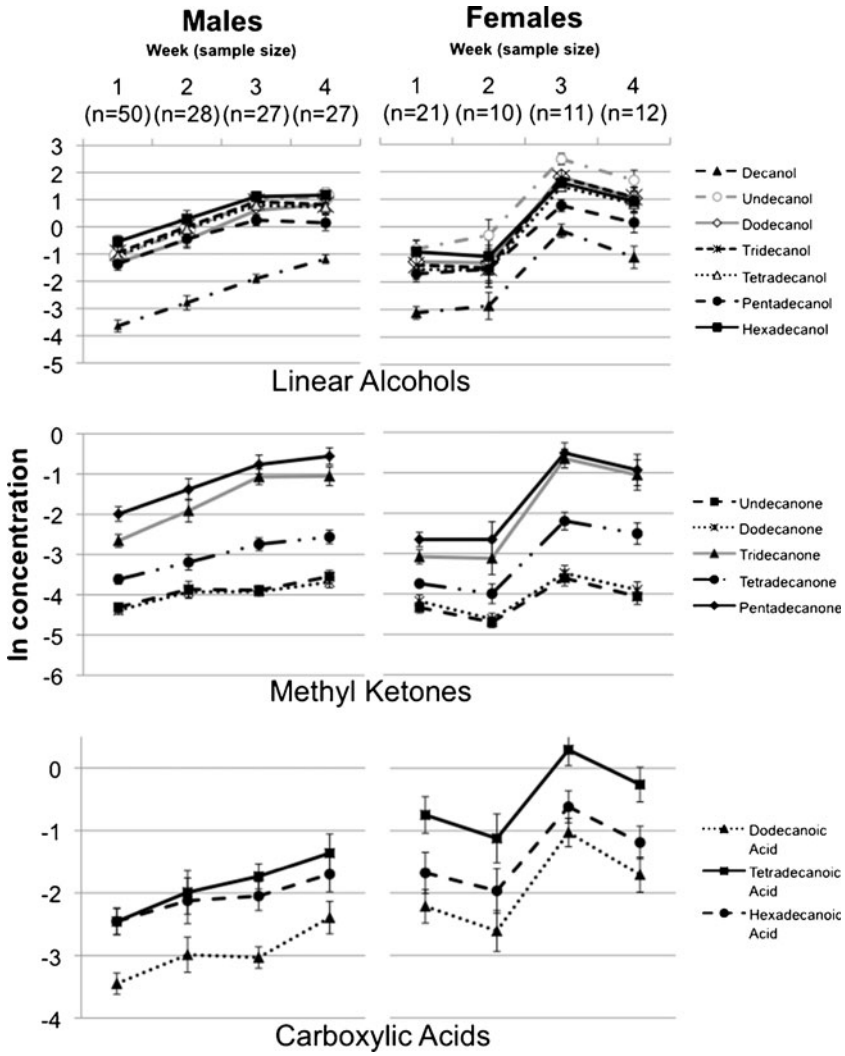


Fig. 1 Mean ln-transformed preen oil volatile compound relative concentrations (relative amounts in 100 μl of preen oil) in juncos, for each week during early season 2008, in males (*left*) and females (*right*). *Error bars* indicate standard error of the mean

(Mann–Whitney *U* test, $P>0.05$, all comparisons), so we combined the data for the two sexes for all subsequent analyses. Changes in volatile compound relative concentrations observed in week 2 were not different among treatment groups (data not shown, $P>0.05$) but, at week 4, birds treated with T showed increases of several volatile compounds (Fig. 3). Control birds also showed increases in many compounds, but these were generally much smaller than observed in T-treated birds. When comparing across the three treatment groups, a dose-dependent response was observed for undecanol (Kruskal-Wallis, $P=0.05$), tridecanol ($P=0.045$), and undecanone ($P=0.011$) (Fig. 4a–c). Because there was not a difference in the response of birds receiving low or high T implants for many of the compounds, we also compared control birds to all birds receiving T implants, regardless of dose. In this comparison, birds receiving T implants had increases in undecanol (Mann–Whitney *U* test, $P=0.015$), dodecanol ($P=0.027$), tridecanol ($P=0.014$), undecanone ($P=0.003$), and hexadecanoic acid ($P=0.045$). As an illustration, Fig. 5 shows the pre-implant and post-implant chromatograms from one individual (a male that received a high T implant). In this individual's preen oil, the absolute amount of the alcohols dodecanol, tridecanol, and tetradecanol increased from 1.3 ng.mg^{-1}, 2.6 ng.mg^{-1} and 6.2 ng.mg^{-1}to 8.3 ng.mg^{-1}, 14.2 ng.mg^{-1}, and 28.8 ng.mg^{-1}, respectively (Fig. 5).

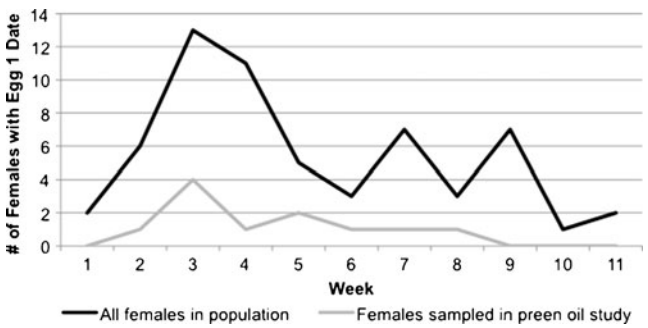


Fig. 2 Date of first egg laid by wild female juncos at Mountain Lake Biological Station, Pembroke, VA in 2008. Weeks correspond to weeks in Fig. 1

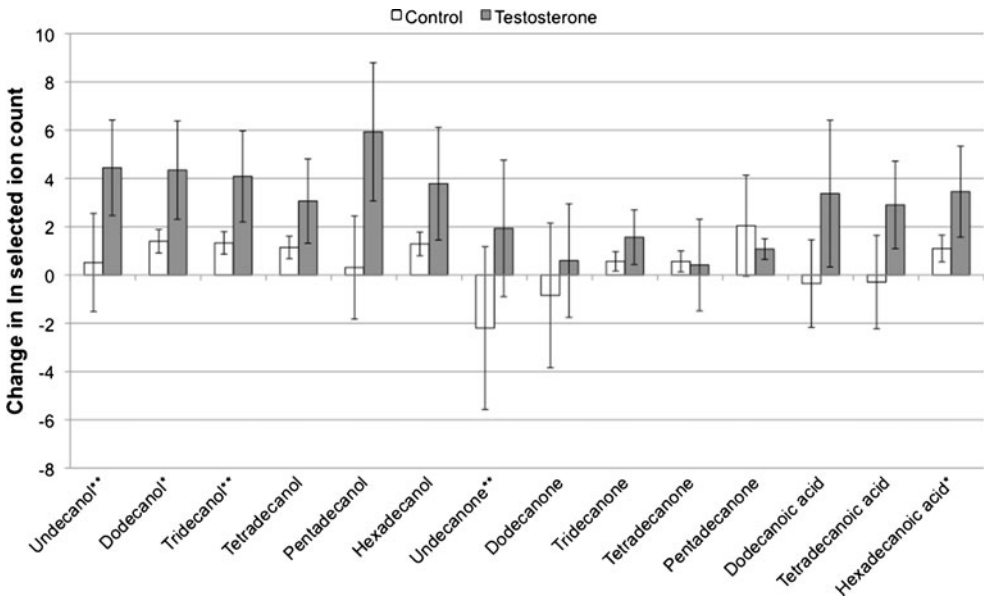


Fig. 3 Changes in ln-transformed volatile compound relative concentrations in juncos 4 weeks after implant with testosterone (T). *Error bars* indicate standard error of the mean. Compounds with increases (Mann–Whitney *U* test, $P<0.05$) in relative concentration in T-implanted birds are noted with an asterisk in the X-axis labels; those that also increased when compared across the three treatments are indicated with two *asterisks*

Discussion

We tested whether testosterone stimulates the production of volatile and semivolatile compounds present in preen oil, and whether the concentrations of these compounds varied in relation to an individual's testosterone levels. We found that volatile compounds increased in concentration during the early breeding season, in tandem with the previously described seasonal increase in circulating levels in T (Ketterson et al., 2005) and seasonal response to GnRH (Jawor et al., 2006, 2007) in wild male and female juncos. Our data showed a concentration peak for females in the same week that most females in the population laid their first egg (week 3), as would be expected if steroid levels influence volatile compound production. In males, we observed a steady increase across the 4 weeks, with volatile compound concentration higher in the fourth than in the first week. Previous studies found that males also experience a drop in T when their mate begins incubating eggs (Ketterson et al. 2005), but the drop is not as sharp as in females. It may be that the males in our study had not yet experienced this decrease in circulating T, or that the decrease was smaller and did not affect volatile compound production. The volatile compounds we observed in wild junco preen oil were the same as those previously described in captive junco preen oil (Soini et al., 2007; Whittaker et al., 2010).

Treatment with exogenous testosterone stimulated the production of some, but not all, preen oil volatile compounds and, for some compounds, birds treated with a high dose of T did not have greater concentrations of preen oil volatile compounds compared to birds that received half the dose. These data suggest that while T in the absence of long days is sufficient to stimulate the production of some volatile compounds, it is not positively correlated with the concentrations of individual compounds. We did not detect differences between the sexes in the implant study, likely due to the fact that both males and females in non-breeding condition are known to display low levels of all preen oil volatile compounds (Soini et al., 2007), and the only manipulation in the study was the circulating level of T. The only volatile compound that is typically in greater concentrations in males than in females, and that responded significantly to T treatment, was 2-undecanone. In another study (Whittaker et al., 2011), we found a positive correlation between the relative proportions of "male-like" volatile compounds (those that make up a greater proportion of the volatile compounds present in male preen oil compared to female preen oil) and a proxy for body size. Thus, there may be another physiological factor involved in producing a male-like chemical profile, and these other factors may not be directly under T control, unlike volatile compounds related to reproductive behavior in rats (Ponmanickam et al., 2010).

Changes in photoperiod affect the production of many different hormones, besides T, which may play a role in stimulating volatile compound production. For example, exposure to light inhibits the production of melatonin from the pineal gland. In mammals, melatonin has been suggested to play a role in regulating the hypothalamic-pituitary-gonadal (HPG) axis, affecting the timing of the production of steroid hormones (Tamarkin et al., 1985). Although melatonin does not appear to be responsible directly for timing reproductive activity in birds, it does play a role in seasonal changes in immune function and song production (Bentley et al., 1999; Bentley, 2001). Thyroid hormone also has been shown recently to play a role in seasonal changes in both mammals and birds (Watanabe et al., 2007). The short photoperiod in the experimental study

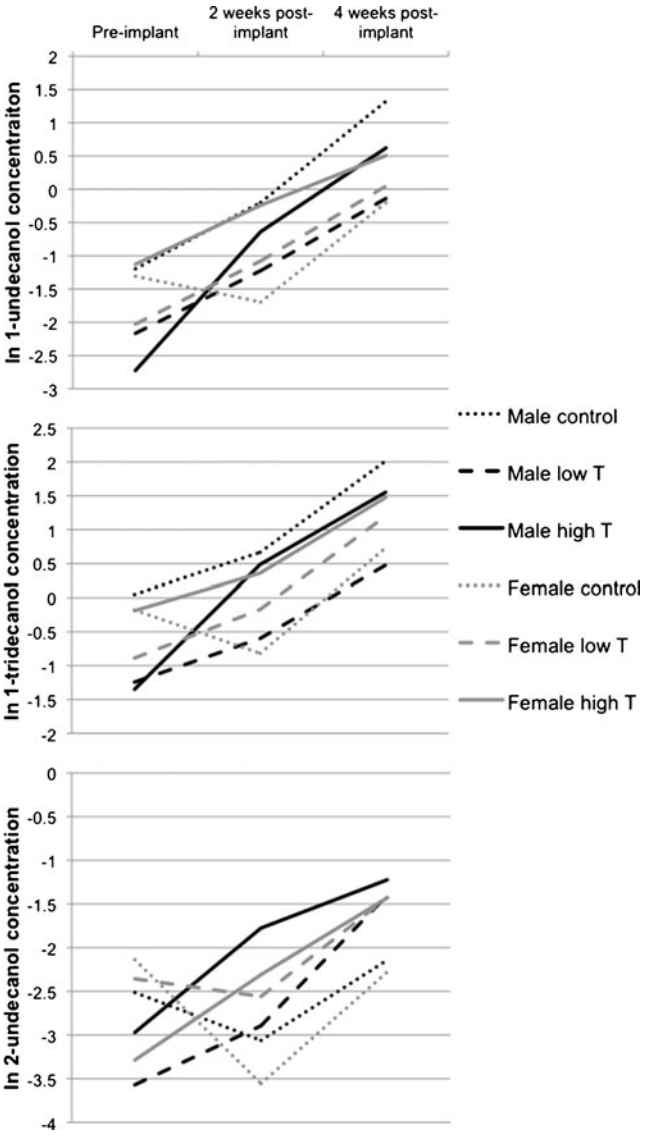


Fig. 4 Average ln-transformed concentrations over time of a) 1-undecanol, b) 1-tridecanol, and c) 2-undecanone in control and testosterone-implanted (at two amounts, low and high) in male and female juncos

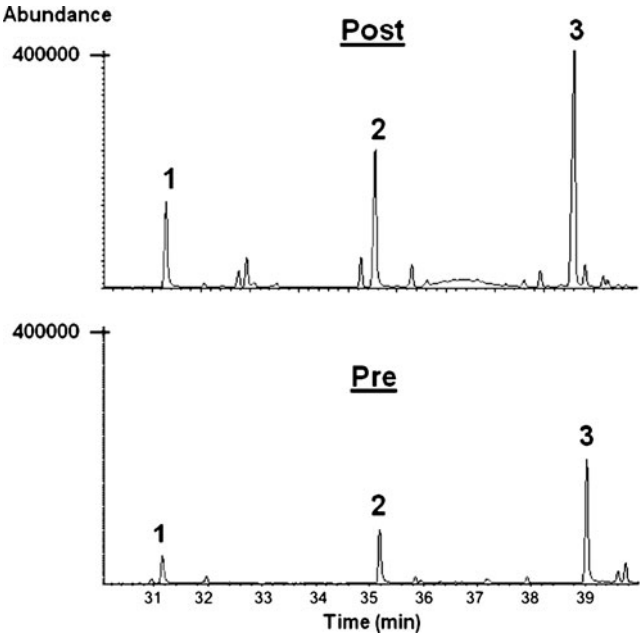


Fig. 5 Selected ion (*m/z* 55) chromatograms (pre- and post-implant), showing comparison of the amounts of selected *n*-alkanols in an individual (ID# 21443) male junco that received a high testosterone implant; 1 = dodecanol (31.1 min), 2 = tridecanol (35.1 min), 3 = tetradecanol (39.0 min)

likely was associated with increased melatonin levels in the birds and decreased levels of the bioactive form of thyroid hormone (T_3), which could have affected the changes in volatile compound concentrations.

The uropygial gland has been described as "androgen-dependent," due to both the high concentration of androgen receptors and the previously observed responses of the gland to treatment with testosterone (Maiti and Ghosh, 1972; Abalain et al., 1983; Amet et al., 1986). In addition to androgen receptors, the uropygial gland contains receptors for other steroid hormones, including receptors for estradiol (Daniel et al., 1977). In order for T to influence target tissues, it must, sometimes, first be converted to an active form, such as dihydrotestosterone (DHT), through the action of 5α-reductase, or estradiol, through the process of aromatization (Massa, 1984). The seasonal changes observed in wild females could have been related to changing estradiol levels, and exogenous T may have been converted to estradiol. Steroid hormone metabolism can take place in the brain to affect behavior (Riters et al., 2001), as well as in peripheral tissues (Edwards et al., 2005), and the abundance of the enzymes involved in this conversion can vary seasonally (Soma et al., 2003). Thus, the inability of our T implant study to replicate volatile compound concentrations observed in breeding birds may be related to differences in concentration of other hormones like melatonin and thyroxine, or a lack of 5α-reductase in males, the quantity of which is known to be decreased in non-breeding birds (Soma et al., 2003).

Other steroid hormones may also play a role in regulating volatile compounds. In the crested auklet, a seabird that produces a citrus-like odorant that does not originate from preen oil, odorant emission was related to levels of the steroid hormone progesterone in males, but not in females (Douglas et al., 2008). Relatively few progesterone, compared to androgen, receptors have been found in the uropygial gland, but progesterone and androgens are known to exhibit cross affinity for receptors (Daniel et al., 1977). Corticosterone, a steroid hormone related to stress, also has been shown to have an effect on cellular activity in the uropygial gland (Maiti and Ghosh, 1969), and corticosterone levels are known to be correlated with T levels in juncos (Ketterson et al., 1991). Thus, a better understanding of the relationship between preen oil volatile

compounds and progesterone or corticosterone levels may be necessary to understand seasonal changes in volatile profiles.

While our results shed light on the role of testosterone on preen oil composition, the role of these volatile compounds in male preen oil remains unknown. Behavioral data suggest female and male juncos are attracted to the scent of males (Whittaker et al., 2011), indicating potential behavioral roles for these compounds. However, their function in females is less clear. The positive relationship between T and "female-like" volatile compounds (those that are typically present in higher proportions in female preen oil) suggests that these compounds could function as a signal of ovulation or receptivity, when T levels are highest. In many species of birds, female T levels peak before or during nest-building (Ketterson et al., 2005), and treatment with T can induce sexual receptivity in many female vertebrates (Adkins-Regan, 1981), most likely as a result of aromatization of T into estradiol (Winkler and Wade, 1998). Data from other avian species also suggest that females produce an olfactory signal that is attractive to males. In domestic chickens, experimental removal of the female's uropygial gland resulted in significantly fewer copulation attempts by males, suggesting that this gland is the source of an attractive signal (Hirao et al., 2009). Female mallard ducks in breeding condition produce preen oil compounds that have been described as pheromones, and these compounds can be induced in nonbreeding males and females by injecting them with exogenous estradiol (Bohnet et al., 1991).

Future research should focus on the action of additional hormones, including estradiol, progesterone, thyroxine, and melatonin, on preen oil volatile compound concentrations. In particular, the relationship between female breeding condition and chemical signals, which is well established in some mammals, should be further investigated in birds.

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level registered in the western Palaearctic region (Enghoff et al., 2011). Defensive secretions from only 13 species in this family have been analzyed (Trave et al., 1959; Schildknecht and Weis, 1961; Röper and Heyns, 1977; Röper, 1978; Huth, 2000). In this study, we identified the constituents of the secretions from three European Julidae species, *Cylindroiulus boleti* (C. L. Koch, 1847) (distributed in Austria, Germany, mainland Italy, eastern Europe, and the Balkan Peninsula), *Leptoiulus trilineatus* (C. L. Koch, 1847) (distributed in mainland Italy, Austria, Switzerland, and the Balkan Peninsula), and *Megaphyllum bosniense* (Verhoeff, 1897) (distributed in Austria, Hungary, mainland Italy, and the Balkan Peninsula) (Enghoff and Kime, 2010).

Methods and Materials

Collection and Handling of Millipedes Individuals of *C. boleti*, *L. trilineatus*, and *M. bosniense* were collected in September-November 2010 at Drugovačka Šuma, near Smederevo, eastern Serbia. The millipedes were stored in plastic boxes with a layer of partly decomposed litter, and kept in the laboratory for a few days at 10°C in the dark. Humidity in the boxes was kept high by spraying water on the litter every day.

Chemical Extraction For the collection of defensive secretions, 15 males and 15 females of each species were soaked in 2 ml of dichloromethane (DCM) for 3 min. To eliminate the effects of composition-altering oxidation and degradation of compounds, a portion of the extracts was analyzed immediately by gas chromatography–mass spectrometry (GC-MS). The rest of each extract was evaporated under nitrogen, and the residue was dissolved in $CDCl_3$ for ^1H NMR spectrometric analysis.

Chemical Analyses and Identification The GC and GC-MS analyses were performed on an Agilent 7890A GC system equipped with a 5975 C inert XL EI/CI MSD and a FID detector connected by capillary flow technology 2-way splitter with make-up gas. An HP-5MSI capillary column (Agilent Technologies, 0.25 mm i.d., 30 m length, 0.25 μm

Table 1 Chemical composition of the defensive secretions in three julid millipede species

Peak No.	Compounds	RI[a]	*C. boleti* [%][b]	*L. trilineatus* [%][b]	*M. bosniense* [%][b]	CI-MS[c] *m/z* (intensity)	EI-MS[d] *m/z* (intensity)
I	1,4-benzoquinone	917	–	–	0.3	109 (100)	108 (M^+, 100), 82 (30), 80 (24), 54 (53)
II	2-methyl-1,4-benzoquinone	1,022	23.9	29.0	38.1	123 (100)	122 (M^+, 100), 94 (81), 82 (51), 66 (46)
III	2-hydroxy-3-methyl-1, 4-benzoquinone	1,127	0.8	4.8	0.4	139 (100)	138 (M^+, 100), 82 (29), 55 (19), 54 (17)
IV	2-methoxy-3-methyl-1, 4-benzoquinone	1,191	70.0	48.8	56.0	153 (100)	152 (M^+, 100), 122 (39), 109 (23), 66 (24)
V	2,3-dimethoxy-5-methyl-1, 4-benzoquinone	1,322	0.8	1.6	1.0	169 (100)	168 (M^+, 78), 153 (37), 123 (100), 69 (49)
VI	2-methylhydroquinone	1,344	0.3	0.7	0.3	125 (100)	124 (M^+, 100), 123 (43), 95 (18), 77 (10)
VII	2-methoxy-5-methylhydroquinone	1,350	0.4	0.6	0.4	155 (100)	152 (M^+, 59), 122 (40), 69 (100), 53 (26)
VIII	2,3-dimethoxyhydroquinone	1,373	0.5	1.1	0.4	171 (100)	170 (M^+, 100), 155 (46), 112 (25), 109 (25)
IX	2-methyl-3,4-methylenedioxyphenol	1,390	2.0	11.2	0.9	153 (100)	153 (9), 152 (M^+, 100), 151 (88), 121 (13)
X	2,3-dimethoxy-5-methyl-1, 4-benzoquinone	1,420	0.2	0.6	0.9	183 (100)	182 (M^+, 68), 167 (36), 137 (100), 83 (53)
XI	2-methoxy-3-methylhydroquinone	1,425	0.7	0.6	0.2	155 (100)	154 (M^+, 100), 139 (62), 111 (46), 65 (12)
XII	2,3-dimethoxy-5-methylhydroquinone	1,455	0.4	1.0	1.1	185 (100)	184 (M^+, 100), 169 (77), 126 (27), 123 (15)

[a] retention indexes obtained using retention times of *n*-alkanes under the same GC conditions

[b] relative abundance computed from GC (FID) peak areas

[c] the most abundant ion represents quasi-molecular ion $[M+H]^+$

[d] four most abundant ions (for EI-MS spectra see Supplemental Figs. S2–S13)

film thickness) was used. Samples were injected in splitless mode, and the injection volume was 1 μl. The carrier gas (He) flow rate was 1.6 ml/min at 40°C (constant pressure mode). The column temperature was programmed linearly in a range of 40–300°C at a rate of 10°C/min, with an initial 1-min and a final 8-min hold. Mass spectra were acquired in electron ionization mode (EI) with ion energy of 70 eV and chemical ionization (CI) mode with ion energy of 150 eV. CI mass spectra were obtained in positive mode with isobutane as the reagent gas. The scan range was *m/z* 35–550 in EI mode, and *m/z* 60–550 in CI mode.

The identification of the compounds was based on comparison of their EI mass spectra with the NIST MS Search 2.0 computerized mass spectral libraries Wiley7 and Nist05, and with the available literature EI-MS data of the compounds previously identified in the defensive secretions of millipedes (Attygalle et al., 1993; Wu et al., 2007). Further, CI-MS spectra of all twelve compounds showed characteristic base peaks at m/z values numerically equal to their respective nominal masses plus one values. The NMR spectra were recorded on Varian Gemini 2000 (200 MHz for ^{1}H) and Bruker Avance III 500 (500 MHz for ^{1}H) spectrometers. Compounds II and IV were confirmed by this method.

Results

From the defensive secretions of three Julidae species (*C. boleti*, *L. trilineatus*, and *M. bosniense*), obtained by whole body extraction in DCM that were analyzed using GC and GC-MS, a total of 12 benzoquinone derivatives were identified: 1,4-benzoquinone (**I**), 2-methyl-1,4-benzoquinone (**II**), 2-hydroxy-3-methyl-1,4-benzoquinone (**III**), 2-methoxy-3-methyl-1,4-benzoquinone (**IV**), 2,3-dimethoxy-5-methyl-1,4-benzoquinone (**V**), 2-methylhydroquinone (**VI**), 2-methoxy-5-methylhydroquinone (**VII**), 2,3-dimethoxyhydroquinone (**VIII**), 2-methyl-3,4-methylenedioxyphenol (**IX**), 2,3-dimethoxy-5-methyl-1,4-benzoquinone (**X**), 2-methoxy-3-methylhydroquinone (**XI**), 2,3-dimethoxy-5-methylhydroquinone (**XII**) (Table 1, Fig. 1). The gas chromatograms of DCM extracts of the three investigated species and the EI-MS spectra of compounds I-XII are shown in Supplemental Figs. S1–S13. The GC and GC-MS data are summarized in Table 1. The relative abundances of the components were computed from the corresponding GC-FID peak areas.

Apart from benzoquinone derivatives, GC chromatograms of the DCM extracts of all three species revealed the presence of other compounds (Supplemental Fig. S1).

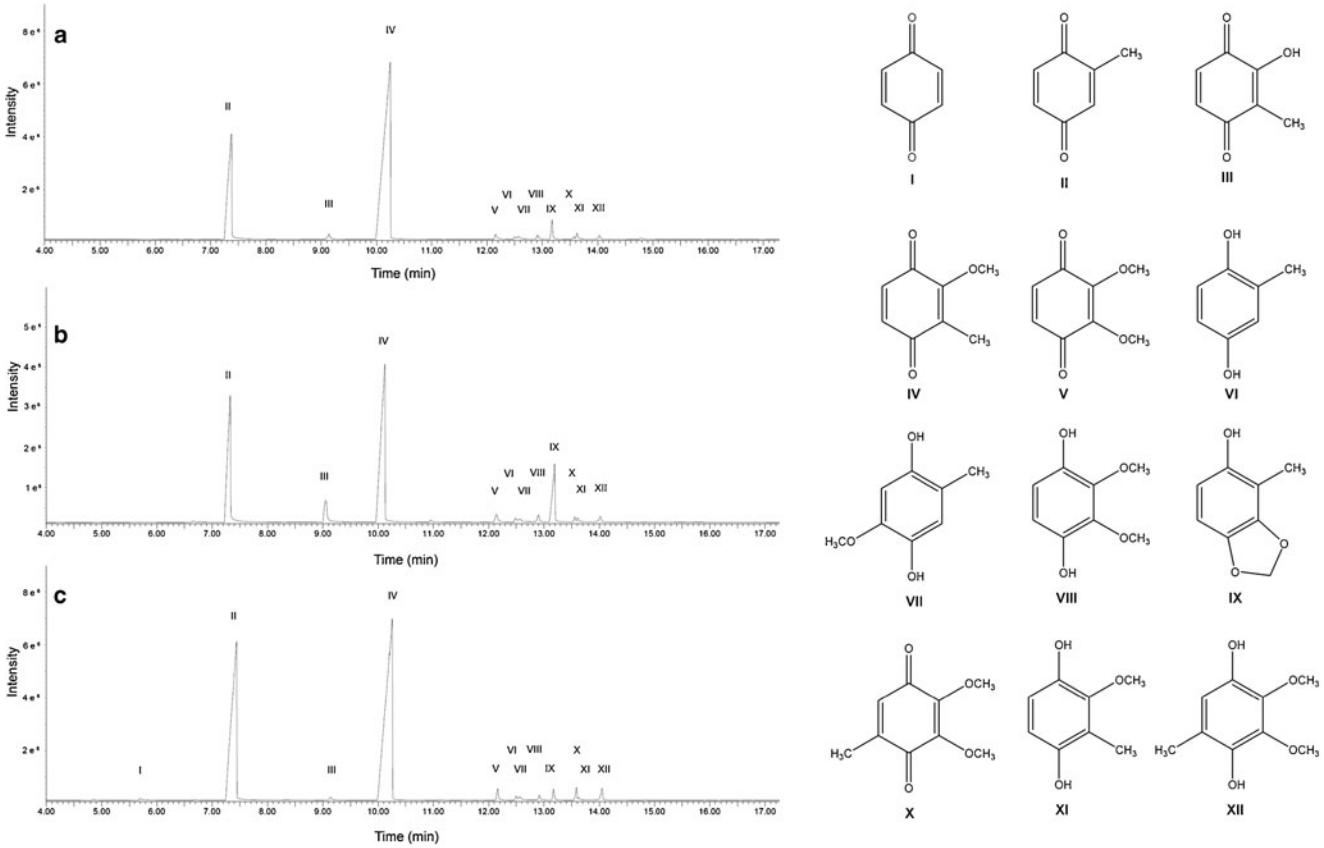


Fig. 1 GC-FID profiles of DCM extracts of julid millipede species *Cylindroiulus boleti* (**a**), *Leptoiulus trilineatus* (**b**), *Megaphyllum bosniense* (**c**), and the structures of identified benzoquinone compounds

Based on their EI and CI mass spectral data, these compounds were identified as hexyl esters of saturated and unsaturated fatty acids with chain lengths from C_{14} to C_{20}. The same compounds were previously detected in the secretions of seven European julids (Huth, 2000), but there is no evidence as to how or whether they contribute to the defensive mechanism of millipedes.

The two main benzoquinone derivatives in all three species were compounds **II** and **IV**, which were structurally confirmed by NMR analysis. In the ^{1}H NMR spectrum of the DCM extract of *M. bosniense* (Fig. 2) and the two other species, the dominant signals arise from hexyl esters of fatty acids detected by GC-MS, but signals characteristic for benzoquinone derivatives also can be observed. Two major doublets at δ 6.69 and 6.60 ppm belong to the most abundant benzoquinone compound **IV**, as do singlets at δ 4.03 ppm (-OCH_3) and 1.95 ppm (-CH_3). Compound **II** is characterized by three benzoquinone protons at δ 6.76, 6.71, and 6.62 ppm, and by a doublet from -CH_3 at δ 2.07 ppm. The ^{1}H and ^{13}C NMR assignments of compounds **II** and **IV**, detailed below, were completed using 2D NMR (HSQC, HMBC, and COSY; Supplemental Figs. S14–S18). These NMR data corresponded well with the literature (Gonzáles et al., 2006; Miyamura et al., 2008), with the exception of the coupling between the -CH_3 group and H-3 of compound **II**. We observed this coupling in the ^{1}H NMR spectrum (Fig. 2), and it was confirmed by COSY correlation (Supplemental Fig. S15), but no such coupling has been reported so far.

2-Methyl-1,4-benzoquinone (**II**): **^{1}H NMR** (500 MHz, $CDCl_3$, *J* in Hz) δ: 6.76 (1H, d, *J*=10, H-6), 6.71 (1H, dd, *J*= 10, 2.5, H-5), 6.62 (1H, dq, *J*=2.5, 1.6, H-3), 2.07 (3H, d, *J*= 1.6, -CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$, ppm) δ: 187.6 (C-1), 183.9 (C-4), 145.9 (C-2), 136.6 (C-5), 136.5 (C-6), 133.3 (C-3), 15.8 (-CH_3).

2-Methoxy-3-methyl-1,4-benzoquinone (**IV**): **^{1}H NMR** (500 MHz, $CDCl_3$, *J* in Hz) δ: 6.69 (1H, d, *J*=10, H-5), 6.60 (1H, d, *J*=10, H-6), 4.03 (3H, s, -OCH_3), 1.95 (3H,

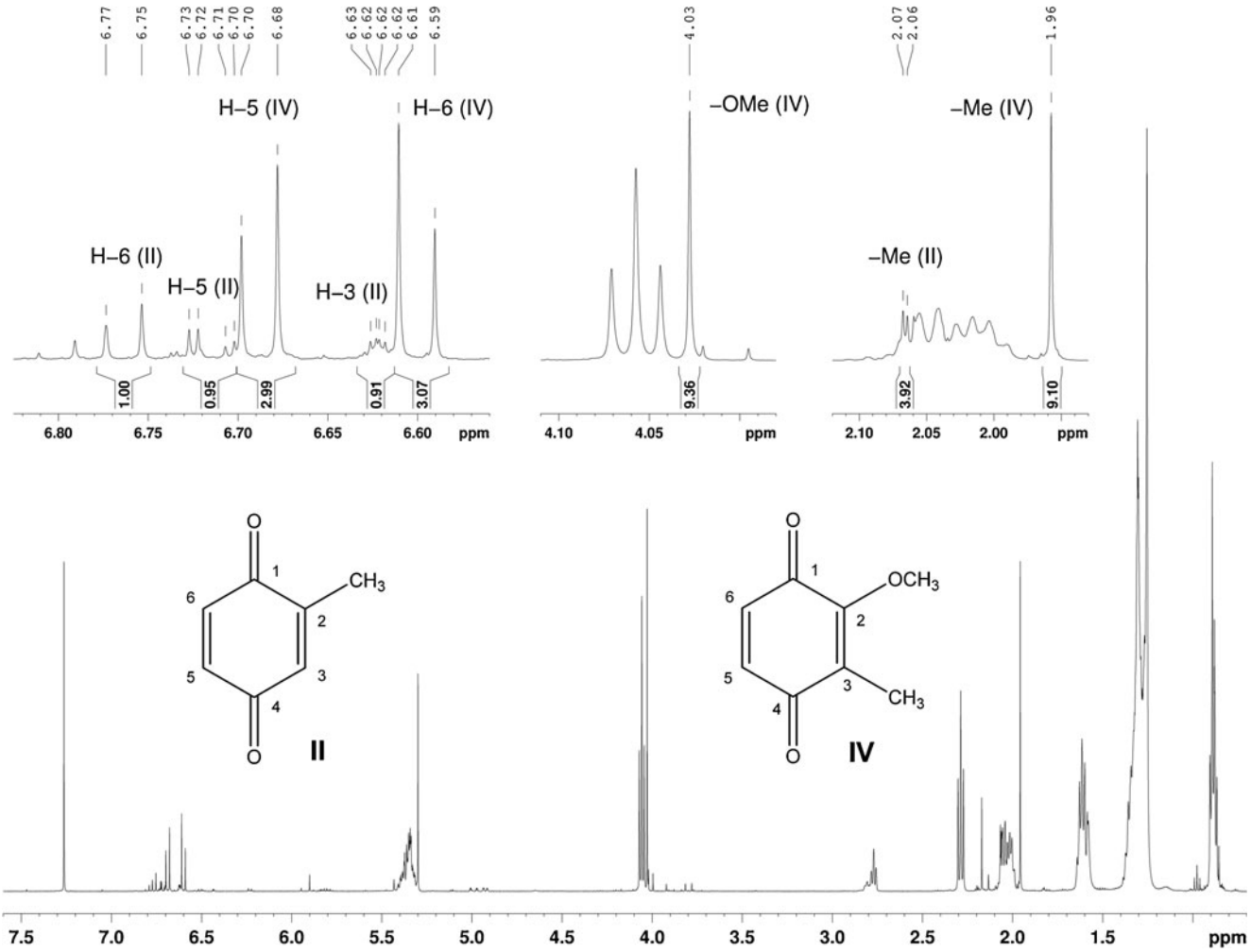


Fig. 2 ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the DCM extract of *Megaphyllum bosniense*; expansions show signals of compounds **II** and **IV**

s, -CH_3); **^{13}C NMR** (125 MHz, $CDCl_3$, ppm) δ: 188.3 (C-4), 183.3 (C-1), 155.7 (C-2), 136.3 (C-5), 134.7 (C-6), 129.0 (C-3), 60.8 (-OCH_3), 8.6 (-CH_3).

Discussion

Cylindroiulus boleti, *L. trilineatus*, and *M. bosniense* possess pairs of repugnatorial glands on nearly every pleurotergite. When disturbed, these millipedes emit the noxious exudates from the glands through openings along the lateral sides of the body. The chemical components from these species are similar, including several benzoquinones and hydroquinones (Table 1). No differences in the composition of the secretions from males vs. females were noted. Moreover, the two main compounds found in the exudates of the analyzed species, 2-methoxy-3-methyl-1,4-benzoquinone (**IV**) and 2-methyl-1,4-benzoquinone (**II**), also are the major components in other julids (Huth, 2000), as well as in some spirobolids (Attygalle et al., 1993; Wu et al., 2007). Generally these two compounds are the most common components of the defensive secretions of juliformian millipedes. In this study, compound **VII** (2-methoxy-5-methylhydroquinone) has been detected for the first time in representatives of the Class Diplopoda, and compounds **III**, **VIII**, **IX**, and **XII** were detected for the first time in members of the family Julidae (Table 2). Within the Juliformia, compounds **IX** and **XII** had been isolated previously only from the defensive fluids of *Acladocricus setigerus* (Silvestri, 1897) (Spirobolida) from Micronesia (Wu et al., 2007). Wu et al. (2007) hypothesized that the oxidative cyclodehydrogenation of 2-methoxy-3-methyl-1,4-benzoquinone leads to the formation of 2-methoxy-3-methylhydroquinone, and that 2-methyl-3,4-methylendioxyphenol is a product of this reaction as well. Our results are congruent with this assumption, since we found all three compounds.

Previous studies (Huth, 2000; Makarov et al., 2010) demonstrated that in some millipedes differences in the defensive secretions are found among the minor components,

Table 2 Chemical composition of the defensive secretion in all analyzed members of the family Julidae. Compounds **I-XII** are listed in Table 1; **XIII**: 2-methoxy-1,4-benzoquinone, **XIV**: 2-methoxy-5-methyl-1,4-benzoquinone, **XV**: 2-ethyl-1,4-benzoquinone, **XVI**: 2-methoxy-6-methyl-1,4-benzoquinone

Compounds Species	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	Reference
Allajulus nitidus (Verhoeff)				+													Röper and Heyns, 1977; Röper, 1978
Cylindroiulus boleti (C. L. Koch)		+	+	+	+	+	+	+	+	+	+	+					This paper
C. caeruleocinctus (Wood)=*C. teutonicus* Pocock	+	+		+	+	+				+	+			+	+	+	Huth, 2000; Röper and Heyns, 1977; Schildknecht and Weis, 1961; Röper, 1978
C. luridus (C. L. Koch)	+			+		+					+						Röper and Heyns, 1977; Röper, 1978
C. meinerti (Verhoeff)	+	+		+	+					+			+	+		+	Huth, 2000
C. punctatus (Leach)	+	+		+	+	+				+	+		+	+		+	Huth, 2000; Röper and Heyns, 1977; Röper, 1978
Enantiulus nanus (Latzel)				+	+					+				+		+	Huth, 2000
Julus scandinavius Latzel	+	+		+	+					+			+	+		+	Huth, 2000
Leptoiulus proximus (Nemec)	+	+		+	+					+			+	+		+	Huth, 2000
L. trilineatus (C. L. Koch)		+	+	+	+	+	+	+	+	+	+	+					This paper
Megaphyllum bosniense (Verhoeff)	+	+	+	+	+	+	+	+	+	+	+	+					This paper
M. unilineatum (C. L. Koch)=*Brachyiulus unilineatus*	+			+													Schildknecht and Weis, 1961
Ommatoiulus sabulosus (Linnaeus)	+	+		+	+					+			+	+	+	+	Huth, 2000; Trave et al., 1959
Ophyiulus pilosus (Newport)	+			+		+					+						Röper and Heyns, 1977; Röper, 1978
Tachypodoiulus niger (Leach)	+	+		+	+					+			+	+		+	Huth, 2000
Unciger foetidus (C. L. Koch)	+			+		+					+						Röper and Heyns, 1977; Röper, 1978

suggesting that chemical patterns in the defensive fluids may be useful in chemotaxonomy. However, our results demonstrate almost identical patterns in the compositions of the defensive secretions from the genera *Cylindroiulus*, *Leptoiulus*, and *Megaphyllum*. We detected only minor intergeneric differences, such as the presence of 1,4-benzoquinone only in *M. bosniense*, and a large quantity of compound **IX** in *L. trilineatus*, suggesting that the secretions have limited chemotaxonomic value (Table 1).

The differences in the chemical defense components between our study and previous work is a result of the general technological advancement in methods for the chemical analysis of defensive fluids, allowing identification of numerous minor substances (Schildknecht and Weis, 1961; Röper and Heyns, 1977; Röper, 1978; vs. this paper). In addition different sampling methods, such as direct extraction from glandular reservoirs (Huth, 2000) vs. whole body extracts (this paper), may lead to different composition data. The chemical compositions of the defensive secretions in the members of the family Julidae are summarized in Table 2. Unfortunately, such differences in the chemical composition of defensive fluids do not allow us to make any comparisons (either among or within genera) or generalizations concerning julid chemotaxonomy.

Our explanation for these differences is supported by the fact that our results are similar to the established chemical composition of Pacific spirobolid millipedes (Wu et al., 2007). Apart from compound **VII**, all the components from our results also are listed by Wu et al. (2007) who used the same sampling method we used in a study of the exudates of *Acladocricus setigerus*. Two compounds (2-ethyl-1,4-benzoquinone and 2-methoxy-3,6-dimethyl-1,4-benzoquinone) were found in this spirobolid that are absent in the present study. Similarities in the defensive compounds in the members of two different orders are probably partly the result of identical sampling methods. The data also support the idea of the chemical consistency of defensive components, at least in the Juliformia taxon (Eisner's "quinone millipedes"; Eisner et al., 1978), as well as the existence of phylogenetically close relationships within juliformian millipedes *sensu lato* (Woo et al., 2007).

A small number of julidean species has been analyzed in comparison to the total number of species, and comparable data often are missing. Further investigations of the defensive chemicals from a wider range of juliformian species could provide more information about chemotaxonomy at different taxonomic levels.

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creates asymmetric genealogies, such that full sisters are more closely related to each other, than they are to their brothers (Trivers and Hare, 1976). This relatedness asymmetry, however, decreases when the queen has mated more than once (polyandry) or if colonies contain several related queens (polygyny) (Boomsma, 1993). Consequently, when kin structure varies among colonies in the same population, theory predicts that workers can enhance their inclusive fitness by specializing on the sex to which they are most related relative to the population average, i.e., females in monogynous/monandrous colonies (with high relatedness asymmetry), and males in polyandrous or polygynous colonies (with low relatedness asymmetry) (split sex ratio theory; Boomsma and Grafen, 1990, 1991). Indeed, empirical studies, mainly of ant species, have found sex allocation patterns consistent with worker control in response to variation in relatedness asymmetry (Chan and Bourke, 1994; Sundström, 1994; Sundström et al., 1996), although other studies have found sex allocation patterns inconsistent with worker control in response to variation in relatedness asymmetry (Brown and Keller, 2000; Liautard et al., 2003; Bonckaert et al., 2011).

Precise adjustment of sex ratios by colony workers in response to colony kin structure requires a mechanism by which they can assess the genetic diversity within colonies, and manipulate brood composition accordingly, e.g., by removing male larvae (Chapuisat et al., 1997). A parsimonious hypothesis is that recognition cues are at least partly genetically determined, so that within-colony recognition cue variability to a certain extent reflects genetic diversity and, thus, relatedness asymmetry. Ants and other social insects use cuticular hydrocarbons (CHCs) for discriminating colony members from strangers to maintain colony integrity. Typically, each colony is characterized by specific proportions of a set of CHCs (reviewed in van Zweden and d'Ettorre, 2010), the exact relative compositions of which can have strong genetic underpinnings (e.g., Stuart, 1988; van Zweden et al., 2009, 2010). On the other hand, if the individuals' blends of CHCs accurately reflect their genetic lineage within a colony, this could allow workers in colonies that contain different patrilines or matrilines to favor close kin to the detriment of more distant kin, and so promote their own evolutionary interests. Such nepotistic discrimination is expected to carry costs with reduced colony efficiency and social disruption in its wake (Keller, 1997), and may lead to selection against highly diverse recognition cues (Ratnieks et al., 2007). Therefore, the level of information encoded in social insect recognition cues is likely to be the result of these two opposing evolutionary forces, being good enough to allow efficient discrimination against non-nestmates but noisy enough to prevent precise discrimination within colonies (van Zweden et al., 2010). Evidence to date indicates that this is indeed the case in the ant *Formica truncorum* (Boomsma et al., 2003) and the wasp *Vespa crabro* (Dani et al., 2004), where CHC variation explained by patriline affiliation is variable among colonies, although a recent study on *Acromyrmex octospinosus* shows higher kin specificity (Nehring et al., 2010).

Not all components of the CHC profile are of equal importance in inter- and intra-colony recognition. Methyl branched alkanes and alkenes have been especially implicated in nestmate recognition, whereas the role of linear alkanes in nestmate recognition is less clear (Dani et al., 2001, 2005; Akino et al., 2004; Greene and Gordon, 2007; Martin et al., 2008; Guerrieri et al., 2009). Conversely, the relative abundance of linear alkanes appears, for example, to be involved in within-colony recognition of (worker) castes in harvester ants (Wagner et al., 2001; Greene and Gordon, 2003; Martin and Drijfhout, 2009). Therefore, we hypothesize that CHCs associated with nestmate recognition may be uniform within colonies, whereas those involved in the assessment of colony kin structure may be more variable (c.f., van Zweden et al., 2010).

In the ant *Formica exsecta*, both the number of queens that head the colony (monogyny vs. polygyny) and the number of males that mate with a queen (monandry vs. polyandry) vary. A previous study showed sex ratio specialization consistent with the predictions from split sex ratio theory (Sundström et al., 1996). This implies that workers can assess colony kin structure also when the relatedness asymmetry varies solely due to queen mating frequency. The CHC profile of *F. exsecta* typically consists of five to six linear alkanes (*n*-C_{21}: *n*-C_{31}) and five to six alkenes ((*Z*)-9-$C_{21:1}$: (*Z*)-9-$C_{31:1}$), of which the latter have been implicated as nestmate recognition cues (Martin et al., 2008). In this study, we compared the CHC variability among workers in monogynous/monandrous, monogynous/polyandrous, and polygynous colonies of *F. exsecta*, to assess whether workers have enough information to act in their best inclusive fitness interests. We predicted that CHC variability would be greater in polyandrous and polygynous than in monogynous/monandrous colonies, thus reflecting lower relatedness asymmetry. The second prediction was that greater CHC variability corresponds to colonies with male-biased sex ratios. We then combined the information on CHC and genetic diversity with sex ratio data for the same colonies to evaluate the degree to which sex allocation corresponds to colony relatedness on the one hand and CHC variability on the other.

Methods and Materials

Study Organisms and General Procedures Workers of F. exsecta were collected from 9 colonies in June 2007 and from 6 colonies in June 2009 (Table 1), on islands off the

Tvärminne Zoological Station, southwestern Finland. The population comprises largely monogynous colonies headed by queens mated with 1 to 3 males, with the exception of a few polygynous colonies (Haag-Liautard et al., 2009). This is the same population where Sundström et al. (1996) found that colonies headed by a singly mated queen specialize in female brood, and those headed by a multiply mated queen specialize in male production. Altogether we chose 5 colonies with a single queen that had mated once (monogynous-monandrous, MG/MA), 5 colonies where the queen had mated multiply (monogynous-polyandrous, MG/PA) and 5 colonies with multiple queens (polygynous, PG) (Table 1). From each colony, 18–24 workers were collected and killed by freezing at −20°C for later genetic and chemical analysis.

Sex ratios of the study colonies were assessed by sampling 50–60 sexual pupae from each colony, in June-July 2007 and 2009 after all the larvae had pupated and before any sexuals had emerged. The sex of the pupae was determined based on morphology, and the sex ratio was calculated as the proportion of queens of all sexual brood (*c.f.*, Vitikainen et al., 2011).

Genetic Analysis To confirm the kin structure of the sampled colonies and to determine patriline or matriline affiliation, the 18–24 workers of each colony were genotyped at ten loci: Fe11, Fe13, Fe17, Fe37, Fe38, Fe42, Fe49 (Gyllenstrand et al., 2002), Fl21 (Chapuisat, 1996), P22 (Trontti et al., 2003), and Fy3 (Hasegawa and Imai, 2004). Further details of PCR conditions are equal to Haag-Liautard et al. (2009). The PCR-products were separated by using automated capillary sequencer (MegaBACE 1000) and sized against ET400-R standard (GE Healthcare). The genotypes were scored with the program Fragment Profiler v1.2 (GE Healthcare), and allele calling was confirmed manually.

We used maximum likelihood methods to confirm the genetic composition of the colonies, and to assign workers into groups of full- and half-sisters, as implemented in the program COLONY 1.3. (Wang, 2004). Average within-colony relatedness was calculated as the pedigree relatedness, based on the equations in Bourke and Franks (1995), assuming r=0.75 for MG/MA colonies, and using the observed paternity shares in MG/PA colonies. For the PG colonies, we inferred the number of matrilines from the genotype data assuming the queens were unrelated (r=0). The true value may be higher, so we ran each test also with higher values without finding any differences in the outcome. Pairwise relatedness estimates between individuals and colonies were calculated based on individual genotypes at each of the ten loci using Relatedness 5.0.8 (Goodnight and Queller, 1999), and colony inbreeding (HL) following the procedure by Aparicio et al. (2006). We used the background population genetic data from 102 colonies (c.f. Haag-Liautard et al., 2009) to enhance the accuracy of allele frequency and relatedness estimates.

Table 1 Basic data for the sampled colonies

Colony	Year	*N*	Sex ratio	Inbreeding coefficient	Gene diversity	Allelic richness	Nr. of matri/ patrilines	Pedigree relatedness	All compounds	(Z)-9-Alkenes	*n*-Alkanes
MG/MA 1	2007	20	0.85	0.29	0.513	2.275	1 m/1p	0.750	1.664	1.021	1.525
MG/MA 2	2007	18	0.17	0.04	0.548	2.279	1 m/1p	0.750	1.676	0.838	1.469
MG/MA 3	2007	19	1.00	0.04	0.513	2.214	1 m/1p	0.750	1.860	0.843	1.214
MG/MA 4	2009	24	0.96	0.26	0.479	2.130	1 m/1p	0.750	1.871	0.423	1.154
MG/MA 5	2009	23	0.00	0.00	0.585	2.462	1 m/1p	0.750	1.365	0.569	1.017
MG/PA 1	2007	20	0.13	0.26	0.516	2.194	1 m/2p	0.503	2.817	1.337	1.562
MG/PA 2	2007	19	0.55	0.39	0.408	1.983	1 m/2p	0.540	1.946	1.191	1.259
MG/PA 3	2007	19	1.00	0.16	0.647	2.805	1 m/2p	0.590	2.701	1.308	1.970
MG/PA 4	2009	24	0.00	0.12	0.577	2.488	1 m/2p	0.674	1.499	0.534	1.074
MG/PA 5	2009	23	0.00	0.11	0.583	2.415	1 m/2p	0.516	1.531	0.277	1.262
PG 1	2007	18	0.15	0.25	0.718	3.373	8 m	0.094	1.940	0.759	1.063
PG 2	2007	19	0.00	0.28	0.622	2.930	7 m	0.107	1.914	0.856	1.368
PG 3	2007	20	0.00	0.28	0.714	3.305	9 m	0.083	2.774	0.848	1.890
PG 4	2009	23	0.29	0.33	0.446	2.180	4 m	0.083	1.475	0.579	1.343
PG 5	2009	23	0.04	0.40	0.604	2.933	7 m	0.048	1.321	0.683	1.129

Colony name also reflects colony type (*MG/MA* monogynous-monandrous, *MG/PA* monogynous-polyandrous, *PG* polygynous). *N* equals the number of individuals screened per colony. Sex ratio is expressed as the number of females as a proportion of the total number of sexual offspring. Pedigree relatedness is calculated based on the assumption that queen relatedness is zero. All compounds, (Z)-9-Alkenes, and *n*-Alkanes refer to cuticular hydrocarbon (CHC) variability measures based on the respective sets of compounds.

Colony-specific gene diversity and allelic richness were calculated with colony defined as population using Fstat 2.9.3 (Goudet, 2001). As the two measures correlated strongly (r=0.94, df=15, P<0.001), we used the factor scores from the first axis in a PCA encompassing the two measures. Comparisons between the different categories of colonies were done with a one-way ANOVA.

Chemical Analysis Cuticular lipids were obtained by individually immersing the 18–24 freshly killed workers (before genotyping them) in 200 µl HPLC-grade pentane for 10 min, with gentle vortexing for 15 sec at the start and end of the 10 min. The pentane was left to evaporate at room temperature under a laminar air-flow hood. Extracts were resuspended in 50 µl pentane, 2 µl of which were injected into an Agilent Technologies 6890 N gas chromatograph (GC), connected to an Agilent Technologies 5975 mass selective detector (MS), using 70 eV electron impact ionization. The GC was equipped with an HP-5MS capillary column (30 m×0.25 mm ID, 0.25 µm film thickness) and a split-splitless injector. The carrier gas was helium at 1 ml/min. After an initial hold of 1 min at 70°C, the temperature rose to 180°C at a rate of 30°C/min, and then to 320°C at 4°C/min with a final hold of 5 min.

The areas of five (*Z*)-9-alkenes and five linear alkanes found on the cuticle of all workers (c.f. Martin et al., 2008) were integrated for further analysis with Agilent ChemStation v. D.02.00.237. Peak areas were normalized for each individual by using a Z-transformation (Aitchison, 1986).

Statistics We estimated within-colony CHC variability both for single compounds and for sets of multiple compounds [all compounds (10 variables), (*Z*)-*9*-alkenes only (5 variables), and linear alkanes only (5 variables)]. For single compounds, we calculated the within-colony standard deviation from the Z-transformed values. For sets of multiple compounds, we first scaled the Z-transformed variables to zero mean and unit variance, and then calculated the absolute Euclidean distance to the mean of the colony. CHC variability then was expressed per colony as the mean distance of its individuals.

To assess which factors may influence within-colony CHC variability, we used ANCOVAs with within-colony CHC variability as the dependent variable, and colony kin structure (MG/MA, MG/PA, PG) or within-colony relatedness, year of sampling (2007 or 2009), within-colony genetic diversity (as captured in the factor scores for gene diversity and allelic richness combined), and colony inbreeding (HL) as the predictor variables. We included HL in the analysis, given that two earlier studies have indicated a significant effect of inbreeding on sex ratios (Haag-Liautard et al., 2009; Vitikainen et al., 2011). To test the effects of within-colony CHC variability, relatedness, genetic diversity, and inbreeding on colony sex ratio we used a multiple regression with stepwise backward elimination. These analyses were done in Statistix 9 (Analytical Software, USA).

To test for an association between genetic and CHC similarity of colonies and individuals within colonies, we created distance matrices, one with the pairwise genetic relatedness based on the ten genotyped loci, and one with the pairwise Euclidean CHC distance. Pairwise Euclidean CHC distance was calculated for sets comprising either all compounds (10 variables), (*Z*)-*9*-alkenes only (5 variables), or linear alkanes only (5 variables). The correlation between the genetic and CHC matrices then was tested using Mantel tests in the program Genodive (v. 2.0b20) (Meiermans, 2010). Correlations between relatedness and CHC distances were performed both between individuals within colonies (i.e., for each colony separately) and between colonies.

Finally, to estimate variation among hydrocarbon profiles explained by patriline or matriline affiliation, we performed a PCA using the MGPA and PG colonies, based on the three sets of multiple compounds [all compounds, linear alkanes only, and (*Z*)-*9*-alkenes only]. This was followed by a MANOVA in the program R (v. 2.12.0) with those PCs explaining >95% of the variation as dependent variables, and colony and patri- or matriline nested within colony as the explanatory variables. We repeated this analysis for each colony separately, except that colony was not used as an explanatory variable in the MANOVA. We corrected the above analyses for multiple testing by using the false discovery rate as appropriate (Benjamini and Hochberg, 1995).

Results

All putative MG/MA colonies were confirmed to be composed of full sisters only. All five MG/PA colonies had two patrilines, and the samples from PG colonies contained between 4 and 9 matrilines (Table 1). The diversity of (*Z*)-*9*-alkenes was in both sampling years significantly lower than that of *n*-alkanes, and the diversity of both was lower in 2009 than in 2007 (full factorial ANOVA, compound type: $F_{1,26}$=46.3, P<0.001, year: $F_{1,26}$= 23.5, P<0.001, compound type x year: $F_{1,26}$=1.08, P=0.31). We found no significant differences in CHC variability between PG, MG/PA, and MG/MA colonies, neither when all compounds were considered together, nor when the subsets of (*Z*)-*9*-alkenes or *n*-alkanes were considered separately [Fig. 1; one-way ANOVA with colony type as explanatory variable: (*Z*)-9-alkenes: $F_{2,12}$=0.57, P=0.58,

n-alkanes: $F_{2,12}$=0.31, P=0.74, all compounds: $F_{2,12}$=0.84, P=0.46]. Similarly, in the more comprehensive model, neither within-colony relatedness, genetic diversity (factor scores for the combined effects of allelic diversity and gene diversity), nor colony inbreeding were significantly associated with CHC variability in any of the three multi-compound sets [all compounds, (*Z*)-*9*-alkenes, *n*-alkanes; Table 2], or any of the individual compounds (Appendix 1). The outcome was qualitatively the same when colony type (PG, MG/PA, and MG/MA) was used instead of within-colony relatedness (Appendix 1). Indeed, within-colony CHC variability was not significantly correlated to any of the other measures, except for sampling year (Table 2). However, genetic diversity did not significantly differ among the three colony types either (PG, MG/PA, MG/MA) ($F_{2,12}$=3.25, P=0.07), suggesting that the increased number of genetic lineages in MG/PA and PG colonies did not necessarily increase genetic diversity.

We found a portion of CHC variation was explained by patri- and matrilines when all compounds or (*Z*)-*9*-alkenes only were considered (all compounds: Wilks' λ=0.333, approx. $F_{148,632.1}$=1.36, P<0.01; (*Z*)-*9*-alkenes: Wilks' λ=0.420, approx. $F_{111,477.1}$=1.44, P<0.01; *n*-alkanes: Wilks' λ=0.521, approx. $F_{111,477.1}$=1.05, P=0.367), although these differences were only pronounced in some of the colonies (Table 3). Matrilines differed significantly in their CHCs in one of the five PG colonies, regardless whether all compounds or only (*Z*)-*9*-alkenes were used. Similarly, in one of the five MG/PA colonies patrilines differed significantly with respect to the (*Z*)-*9*-alkenes (all corrected for false discovery rate; Table 3). We also found a negative correlation between pairs of colonies for pairwise relatedness and (*Z*)-*9*-alkene distance after correction for false discovery rate (r=−0.342, P=0.001), but not for *n*-alkanes or when all compounds were considered (r=−0.021, P=0.401, and r=−0.213, P=0.016, respectively) (Fig. 2). This shows that the relative abundance of (*Z*)-*9*-alkenes has a strong genetic component with respect to between-colony variation. However, within colonies, we found no significant associations between genetic and CHC distances for any of the considered compound sets (Appendix 2).

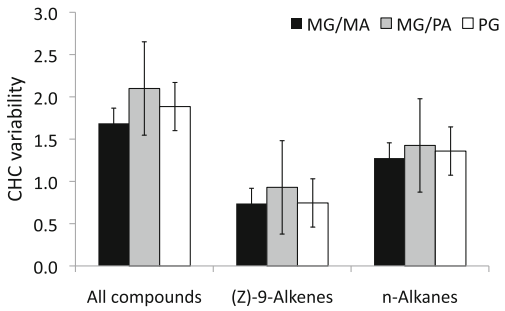


Fig. 1 CHC variability, colony type and CHC group (mean ±95%CI; *MG/MA* monogynous-monandrous, *MG/PA* monogynous-polyandrous, *PG* polygynous)

Table 2 Ancova results for effects on CHC variability

Effect	All compounds		(*Z*)-*9*-Alkenes		*n*-Alkanes	
	F	*P*	*F*	*P*	*F*	*P*
Year	5.63	0.04	20.09	0.001	3.71	0.08
Relatedness	0.12	0.73	0.33	0.58	0.01	0.92
Genetic diversity	0.39	0.55	0.12	0.73	0.07	0.80
Inbreeding	0.25	0.63	1.64	0.23	0.06	0.81

Within-colony relatedness was calculated assuming zero relatedness among queens, and genetic diversity as the combined effects of allelic richness and gene diversity captured in their factor scores. *df*1=1, *df*2=10 in all cases.

The sex ratios of all PG colonies were highly male biased, as expected based on their low relatedness (Fig. 3). The monogynous colonies were more variable. Two of the five MG/PA colonies produced a female biased sex ratio, and two of the five MG/MA colonies produced mainly males (Fig. 3). When the effects of colony-specific CHC variability on sex ratio were considered in conjunction with within-colony relatedness and colony inbreeding (HL), the only significant determinant of colony sex ratios was within-colony relatedness (full model including relatedness and HL as explanatory factors: R^2=0.40, $F_{2,12}$=4.04, P=0.045; HL: $F_{2,12}$=3.13, P=0.102; relatedness $F_{2,12}$=8.07, P=0.015; all other variables, year, all compounds, (*Z*)-*9*-alkenes, *n*-alkanes: $F_{2,12}$<0.35, P>0.50).

Discussion

In this study, we showed that in *Formica exsecta* the within-colony CHC variability is not contingent on colony kin structure, yet genetically related colonies have more similar chemistry, showing a genetic component to the cuticular chemistry without a direct link with the genetic composition of the colony. Nonetheless, genetic lineages were chemically distinct in some but not all colonies. This suggests that chemical cues by which workers can assess colony kin structure are occasionally available, but that these cues generally are unreliable, so that workers would make too many mistakes when using them as a proxy for the sex ratio to be produced by the colony.

Polygynous and monogynous/polyandrous colonies showed no higher amounts of CHC variability than monogynous/monandrous ones. This is in agreement with the result obtained by S.J. Martin et al. (unpublished data) for the same species, but stands in stark contrast with the general expectation of greater CHC variability in genetically more heterogeneous, i.e., polygynous and/or mono-

Table 3 Discriminant analyses between genetic lineages within MGPA and PG colonies

Colony	All compounds (3 PCs)			*(Z)-9*-Alkenes (2 PCs)			*n*-Alkanes (2 PCs)		
	Wilks' λ	*F*	*P*	Wilks' λ	*F*	*P*	Wilks' λ	*F*	*P*
All colonies	0.333	$F_{148,632}=1.36$	0.007	0.420	$F_{111,477}=1.44$	0.005	0.521	$F_{111,477}=1.05$	0.367
MG/PA 1	0.795	$F_{3,16}=1.38$	0.285	0.891	$F_{2,17}=1.05$	0.373	0.858	$F_{2,17}=1.41$	0.272
MG/PA 2	0.502	$F_{3,14}=4.63$	0.018	0.810	$F_{2,15}=1.76$	0.206	0.577	$F_{2,15}=5.49$	0.016
MG/PA 3	0.730	$F_{3,15}=1.85$	0.182	0.711	$F_{2,16}=3.25$	0.065	0.851	$F_{2,16}=1.40$	0.276
MG/PA 4	0.732	$F_{3,20}=2.44$	0.094	0.539	$F_{2,21}=8.99$	0.002	0.829	$F_{2,21}=2.16$	0.140
MG/PA 5	0.718	$F_{3,19}=2.49$	0.092	0.821	$F_{2,20}=2.18$	0.140	0.902	$F_{2,20}=1.09$	0.355
PG 1	0.098	$F_{20,20.7}=1.23$	0.323	0.482	$F_{14,16}=0.50$	0.899	0.221	$F_{14,16}=1.29$	0.310
PG 2	0.290	$F_{18,28.8}=0.88$	0.606	0.255	$F_{12,22}=1.80$	0.113	0.325	$F_{12,22}=1.38$	0.246
PG 3	0.220	$F_{24,26.7}=0.76$	0.746	0.413	$F_{16,20}=0.70$	0.767	0.379	$F_{16,20}=0.78$	0.690
PG 4	0.088	$F_{9,41.5}=7.92$	<0.001	0.023	$F_{6,36}=33.52$	<0.001	0.468	$F_{6,36}=2.77$	0.026
PG 5	0.323	$F_{18,40.1}=1.09$	0.394	0.351	$F_{12,30}=1.72$	0.111	0.436	$F_{12,30}=1.29$	0.277

A PCA was performed on the Z-transformed CHC data of either all colonies together or single colonies; PCs explaining >95% of the variation were then included as the dependent variable in a MANOVA with genetic lineage (nested within colony, when all colonies were considered together) as the explanatory variable.

gynous/polyandrous societies, which has formed the foundation for assuming lower discrimination abilities in polygynous societies (e.g., Fletcher and Michener, 1987; Starks et al., 1998). However, we also found that the within-colony genetic diversity was not higher in the polygynous and the monogynous/polyandrous colonies than in the monogynous/monandrous ones. Indeed, although within-colony relatedness varied 15-fold, the corresponding ranges for both gene diversity and allelic richness were only 1.7 fold in both cases (Table 1). Thus, the genetic basis for a greater diversity is not present, which may explain the lack of correlation between colony kin structure and CHC variability.

The (*Z*)-*9*-alkenes overall were less variable within colonies than *n*-alkanes. This supports the existing evidence that mainly alkenes mediate nestmate recognition in *F. exsecta*, whereas alkanes are less informative in this respect (Martin et al., 2008). Moreover, the between-colony CHC distance for (*Z*)-*9*-alkenes, but not the *n*-alkanes, decreased significantly with increasing between-colony pairwise relatedness. This, in conjunction with the result that within-colony CHC variability showed no clear association with genetic diversity, suggests a strong genetic foundation of the (*Z*)-*9*-alkene profile that is to a large extent blurred within colonies by odor cue transfer between nestmates (c.f. van Zweden et al., 2010). It also suggests unequal mixing

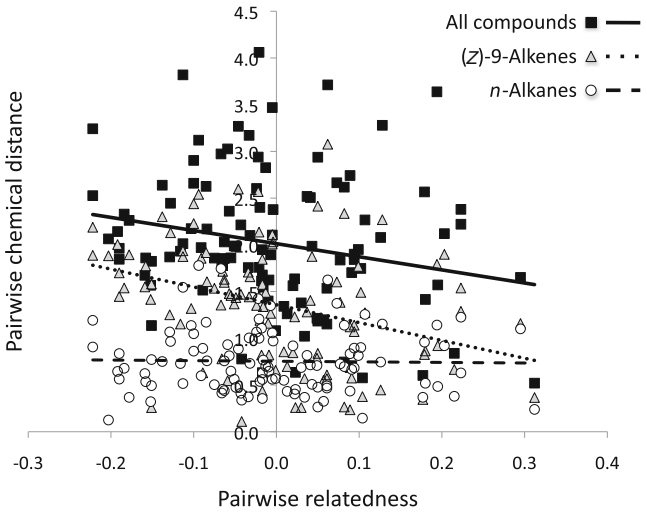


Fig. 2 Correlations between colony pairwise relatedness and colony CHC distances. Association is significant, after correction for false discovery rate, for *(Z)*-9-alkenes (Mantel's test; *(Z)*-9-alkenes, $r=-0.342$, $P<0.001$), but not when *n*-alkanes or all compounds were considered (*n*-alkanes, $r=-0.021$, $P=0.401$; all compounds, $r=-0.213$, $P=0.016$)

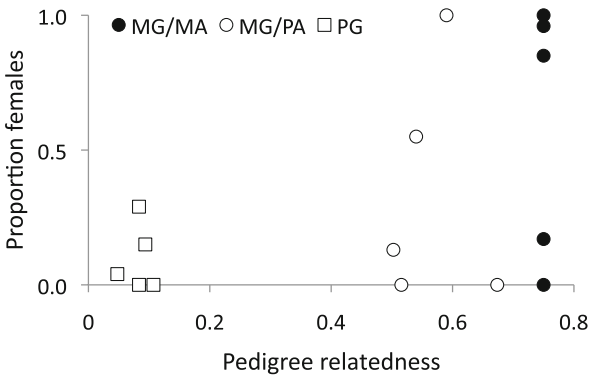


Fig. 3 Sex ratios (numerical proportion of females in the brood) as a function of within-colony relatedness, grouped according to colony type (*MG/MA* monogynous-monandrous, *MG/PA* monogynous-polyandrous, *PG* polygynous)

of CHCs, which could be achieved by accumulating alkenes to a greater extent than alkanes in the postpharyngeal gland (PPG; Soroker et al., 1994). The PPG does not synthesize hydrocarbons, but acts as a reservoir for these compounds to be homogenized and exchanged via grooming and trophallaxis (Soroker et al., 1994). Thus, inequalities in diversity could arise if alkenes are released more readily, or workers metabolize alkanes more rapidly, with alkenes becoming better mixed than alkanes among colony members. Altogether, these results suggest a pattern similar to that observed in *F. rufibarbis*, where cues used in nestmate recognition are genetically determined but preferentially mixed among nestmates (van Zweden et al., 2010).

Although the within-colony CHC variability showed no correlation with colony kin structure, relatedness, or any of the measures of genetic diversity, we found significant CHC divergence between genetic lineages within colonies. This was, however, only the case in two of the ten colonies that comprised more than one lineage. Earlier analyses in the wasps *Polistes dominulus*, *Vespa crabro* (Dani et al., 2004), and *Dolichovespula saxonica* (Bonckaert et al., 2011), and the ant *Acromyrmex octospinosus* (Nehring et al., 2010), found more consistent CHC differences between matrilines and patrilines. However, the CHC profile of *F. exsecta* exhibits far fewer hydrocarbons with fewer structural groups than these species (*P. dominulus*: 44 compounds; *V. crabro*: 25 compounds; *D. saxonica*: 56 compounds; *A. octospinosus*: 38 compounds), and so offers less scope for both divergence and statistical discrimination between genetic lineages. However, in *F. truncorum*, Boomsma et al. (2003) nonetheless were able to discriminate a greater fraction of patrilines although the analysis entailed only nine compounds.

At least two mutually non-exclusive explanations for the relative lack of CHC discrimination among genetic lineages are possible. First, active cue scrambling may render the colony odor more uniform or blur the recognition code. This may be an evolved response that either mitigates the opportunities for workers to selfishly favor close kin and disfavor more distant kin with social disruption in its wake, or increases discrimination between members of different colonies (Keller, 1997; van Zweden et al., 2010). Theory predicts depauperate CHC profiles and cue mixing when potential conflicts are rife in the colony (e.g., Sundström and Boomsma, 2001). This is indeed the case in *F. exsecta*, but many more compounds have been found in *F. truncorum* (Akino, 2006), which goes against this interpretation. Second, given that inbreeding is rife in the population of *F. exsecta* studied here (Sundström et al., 2003; Haag-Liautard et al., 2009; Vitikainen et al., 2011), genetic variation for cue diversity may have been purged. We found no significant effects of inbreeding on CHC variability, but the measure we used as a covariate in the analyses does not reflect the absolute level of inbreeding, only the relative level. Thus, a significant effect of inbreeding may not be detectable.

Within-colony CHC variability had no significant predictive value on sex ratio, but colonies with higher relatedness produced more females, when also accounting for colony inbreeding. This suggests that workers do not use the CHC variability of adult individuals as a proxy for sex ratio decisions, in contrast to the results obtained by Boomsma et al. (2003) for *F. truncorum*. It begs the question by what mechanism this is achieved. All polygynous colonies produced a male-biased brood, but against expectations based on the kin value of brood, one polyandrous colony produced only female brood and one monandrous colony only male brood. In the case of polyandrous colonies, errors in the assessment of colony kin structure may occur if the two fathers are closely related, and so mediate similar CHC profiles to their offspring. However, the polyandrous colony that produced an all-female brood had the lowest relatedness between fathers, and not an exceptionally unequal contribution by the two fathers (Table 1). It remains to be shown what information the workers use to assess colony kin structure, and why such apparent errors occur. A possibility is that CHC information on eggs or larvae more accurately reflects the genetic make-up of the colony, owing to no or less transfer of cues between genetic lineages, and that nurse workers bias the sex ratio accordingly. Another possibility is that nurse workers can assess the number of queens directly or by colony egg production, and use this as a proxy to adjust sex ratio in polygynous colonies. This is consistent with split-sex ratio theory (Boomsma, 1993), because polygynous colonies will tend to have higher egg-production and a lower than population-level-average relatedness asymmetry, and also with the idea that specialization in the cheaper sex (males weigh less in *F. exsecta*; Vitikainen et al., 2011) requires greater initial egg-production for equal total biomass production. On the other hand, this does not explain the tendency of monogynous/polyandrous colonies to specialize in male production. Environmental factors also are likely to influence the optimal sex ratio for a colony, and hence may explain the discrepancies between the observed pattern and the outcome expected based on purely inclusive fitness arguments (Nielsen et al., 1999; Liang and Silverman, 2000).

In summary, we found no support for the suggested association between colony kin structure and variability of recognition cues (c.f. Fletcher and Michener, 1987), but this lack of relationship may be explained by the uniform levels of genetic variation we found among the different colony types. The breeding patterns and the genetic structure of the population, which indicate non-trivial levels of inbreeding (Sundström et al., 2003; Haag-Liautard et al., 2009; Vitikainen et al., 2011), may have contributed to the

depauperate chemical profile through genetic purging, a question which will have to be addressed in future studies. We also found that the within-colony CHC variability does not allow accurate assessment of colony kin structure, and that workers do not seem to use any single compound or set of compound as a proxy for sex ratio adjustments. Rather, genetically informative cues appear to be mixed among workers to create a colony odor that is used in between-colony discrimination. The cuticular hydrocarbon variability of *Formica exsecta* may thus be subject to balancing selection on the accuracy of genetic variability, allowing colony members to serve their collective but not selfish fitness interests, possibly blended with loss of genetic diversity owing to inbreeding in this population.

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each species was cultured on potato dextrose (PD) agar. Isolations of single-hypha strains of all ambrosia-like morphotypes (*Ambrosiella*, *Ambrosiozyma*, or *Raffaelea*) followed previously published protocols (Kolarik and Hulcr, 2008). Morphotyping to genus was confirmed by amplification of ITS1 or SSU rDNA sequences using the primers ITS1 and ITS4, or NS1 and NS4, respectively (White et al., 1990; Gardes and Bruns, 1993), followed by identification in NCBI-BLAST (see Table S1 for results).

Behavioral Assays Using a 2-choice Teflon olfactometer (Analytical Research Systems, Gainesville, FL, USA) thoroughly described in Mann et al. (2011), we measured responses of beetles to volatiles from *R. lauricola* (symbiont of *X. glabratus*), *Ambrosiella xylebori* (symbiont of *X. crassiusculus*), *Ambrosiozyma ambrosiae* (symbiont of *X. ferrugineus*), *Trichoderma* sp. (antagonist of fungi and insects common in ambrosia beetle galleries), and ethanol, a known attractant of many ambrosia beetles. Fungus odor sources consisted of individual 0.25 cm^2 pieces of actively growing mycelium on PD agar medium; sterile medium was used as blank control. In case of ethanol, 1 ml of absolute alcohol was pipetted onto 5 cm of cotton wick; an untreated cotton wick was used as a blank control. A constant airflow of 0.1 l/min was maintained through both olfactometer arms, and a 0.5 l/min suction flow vacuumed the odor mixture from the olfactometer. The olfactometer was housed within a temperature controlled room (26±1°C, 60±2% RH) inside a fiber board box for uniform light diffusion from a fluorescent 900 lx light bulb. We confirmed the lack of positional bias by exposing a batch of beetles to two clean air treatments (blank odor fields).

Approximately 20 newly emerged beetles of a single species were assayed per replicate; each species replicated at least 5 times, always with new beetles. An odor source was randomly assigned to one of the olfactometer arms at the beginning of each assay and reversed after every replication. After each run, the olfactometer and the glass tubes were washed in soapy water and rinsed with distilled water. The glass tubes then were rinsed with acetone and oven dried overnight at 93.3°C, and the olfactometer stage was cleaned with absolute alcohol and air-dried. The numbers of beetles choosing the control arm vs. the treatment arm were compared with the *Chi square* test. Beetles not moving normally (exhibiting difficulty of walking or in poor condition) were excluded from analysis.

Results

We isolated diverse communities of fungi and yeasts from beetle mycangia (for details see Table S1). *Xyleborus glabratus* consistently yielded *Raffaelea lauricola* and *R. subalba* (Ascomycota, Ophiostomatales). *Xyleborus ferrugineus* commonly yielded *Raffaelea albimanens* (Ascomycota, Ophiostomatales), *Ambrosiozyma ambrosiae* (Ascomycota, Saccharomycetales), and an unidentified *Ambrosiozyma*-like strain. We selected *A. ambrosiae* as the experimental symbiont. *Xylosandrus crassiusculus* yielded *Ambrosiella xylebori/hartigi* (ambiguous BLAST assignment; Ascomycota, Microascales). We did not isolate symbionts of *Xyleborinus saxesenii*. To test responses of beetles to a non-symbiotic antagonistic fungus, we chose *Trichoderma* sp. isolated from our samples.

Beetle Responses Each beetle species for which a symbiont was available showed statistically significant attraction to its respective symbiont (Fig. 1). The two representatives of the genus *Xyleborus* were also attracted to certain symbionts from other beetle species (Fig. 1, Table 1). The non-symbiotic *Trichoderma* never triggered attraction from any beetle species. Ethanol was attractive to *X. ferrugineus* and *X. crassiusculus*, and did not elicit significant response from *X. glabratus* and *X. saxesenii* (Fig. 1).

Discussion

The tested fungus-farming ambrosia species responded positively to volatiles from their symbiotic fungi. Specificity of the reaction varied by beetle genus. In both *Xyleborus* spp., we observed attraction to several ambrosia fungi, but *Xylosandrus crassiusculus* was attracted only to its own symbiont. *Xyleborus* spp. are often associated with more symbionts than *Xylosandrus* spp. (Six et al., 2009; Harrington et al., 2010). We suggest that symbiont specificity and odor-perception specificity may be correlated traits. Attraction of ambrosia beetles to volatile cues from their fungal symbionts may function as a mechanism to locate established fungal gardens of conspecific beetles (suitable microhabitat), but also as an orientation cue within a gallery.

Our results are not consistent with Hanula et al. (2008), who did not observe attraction of *X. glabratus* to trees infested with its symbiont as compared with non-infested trees. The difference may have been caused by the unknown vigor of the fungal colonies in the logs exposed for several months in Hanula et al., or perhaps because fungal odors do not transpire readily from the inside of logs. The beetle responses to ethanol, in general, confirmed previous reports (Hanula and Sullivan, 2008; Ranger et al., 2010), except in *Xyleborinus saxeseni*.

Responses of beetles to odors of fungal symbionts were statistically significant, but often weak (Fig. 1). Further experiments may require a modified assay (ambrosia beetles are poor walkers), varying release rates of attractants, and a

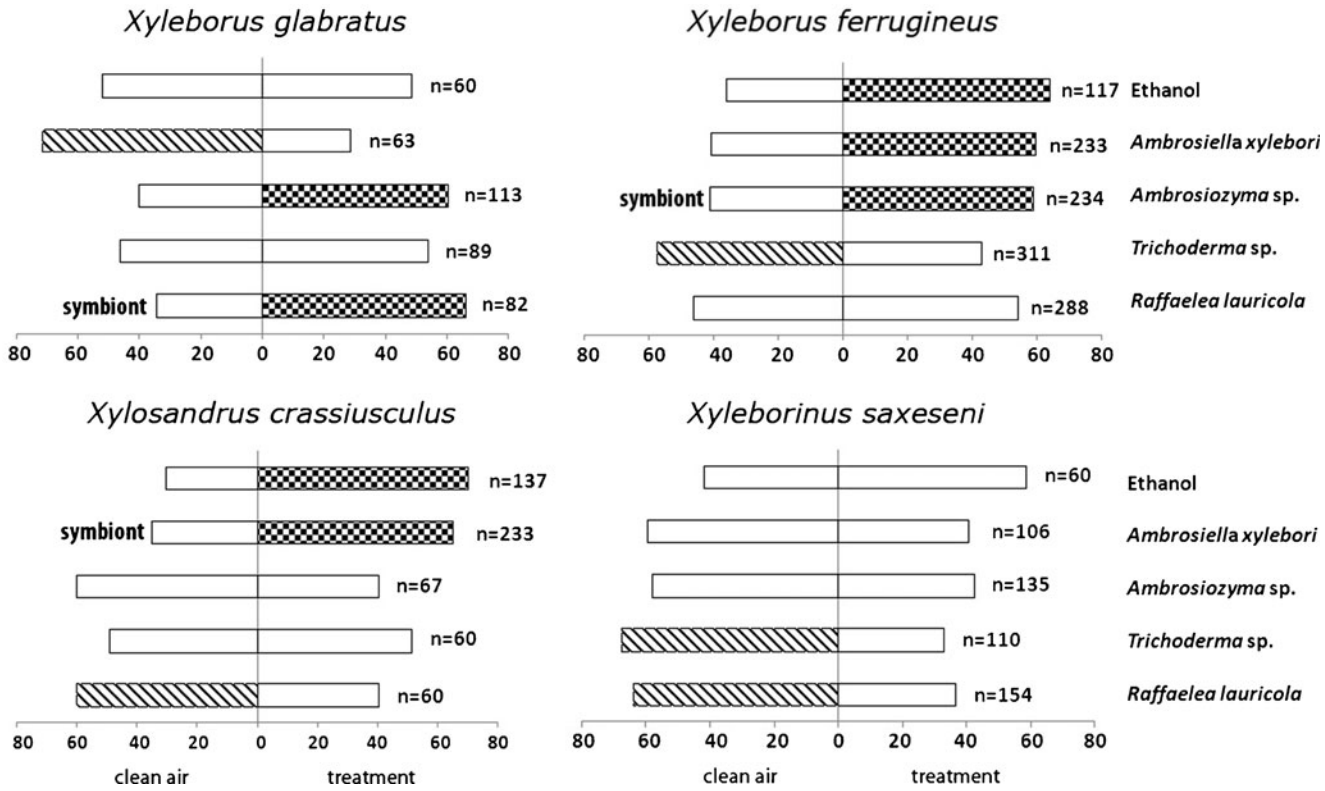


Fig. 1 Percentage of beetles attracted to fungal odors (*right bars*) vs. blanks (sterile agar media, *left bars*), and total numbers of tested individuals. Non-moving beetles were excluded. *Highlighted bars* indicate statistically significant differences (*stripes*: repulsion, *squares*: attraction). The symbiont of each beetle is highlighted (*X. saxesenii* did not have a symbiont in the study)

test of synergy with host tree volatiles. Our results indicate that fungal symbiont volatiles are a source of attractants, with a potential for development of lures for monitoring invasive ambrosia beetles.

Table 1 Responses of ambrosia beetles to volatiles from ambrosia fungi, antagonistic fungus, or ethanol. Fractions: absolute numbers of "beetles in treatment arm/beetles in control arm" summed across all trials. NA: non-active beetles

Beetle species	Response to:				
	Raffaelea lauricola	*Ambrosiozyma sp.*	*Ambrosiella xylebori*	*Trichoderma*	*ethanol*
Xyleborus glabratus	attracted SYMBIONT 54/28, $\chi2$=8.24; df = 1; *P*=0.004, NA = 36	attracted 68/45, $\chi2$=5.05; df = 1; *P*=0.02, NA = 57	repelled 18/45, $\chi2$=11.57; df = 1; *P*=0.001, NA = 19	no response 48/41, $\chi2$=0.55; df = 1; *P*=0.46, NA = 78	no response 29/31, $\chi2$=1.90; df = 1; *P*=0.27, NA = 32
Xyleborus ferrugineus	no response 156/132, $\chi2$=2; df = 1; *P*=0.16, NA = 98	attracted SYMBIONT 138/96, $\chi2$=7.54; df = 1; *P*=0.006, NA = 35	attracted 139/94, $\chi2$=8.69; df = 1; *P*=0.003, NA = 8	repelled 133/178, $\chi2$=6.51; df = 1; *P*=0.01, NA = 102	attracted 107/60, $\chi2$=13.23; df = 1; *P*<0.001, NA = 33
Xylosandrus crassiusculus	repelled 55/82, $\chi2$=5.32; df = 1; P =0.02, NA = 116	no response 27/40, $\chi2$=0.02; df = 1; *P*=0.89, NA = 12	attracted SYMBIONT 39/21, $\chi2$=5.4; df = 1; *P*=0.02, NA = 6	no response 119/114, $\chi2$=0.11; df = 1; *P*=0.74, NA = 102	attracted 42/18, $\chi2$=9.40; df = 1; *P*=0.002, NA = 5
Xyleborinus saxesenii	repelled 56/98, $\chi2$=11.45; df = 1; P =0.001, NA = 50	no response 57/78, $\chi2$=3.23; df = 1; *P*=0.07, NA = 51	no response 43/63, $\chi2$=3.77; df = 1; *P*=0.05, NA = 54	repelled 36/74, $\chi2$=13.13; df = 1; *P*<0.001, NA = 53	no response 34/26, $\chi2$=1.07; df = 1; *P*=0.3, NA = 20

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